UNIVERSITY OF ALBERTA

FREEZE/THAW PROCESS FOR MICROORGANISMS REDUCTION

by

YUN LI ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment

of the requirements for the degree of MASTER OF SCIENCE

in

ENVIRONMENTAL ENGINEERING

DEPARTMENT OF CIVIL AND ENVIRONMENTAL ENGINEERING

EDMONTON, ALBERTA

SPRING, 2005

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

0-494-08129-5

Your file Votre référence
ISBN:
Our file Notre reference
ISBN:

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

DEDICATION

To my wife Dong Li and my son Andrew

for their love and support

Abstract

In this project, the effect of freeze/thaw process on *E.coli* and spores of *Bacillus megaterium* survival was studied. The storage time, the concentration of salt and preserve temperature were the significant factors affecting *E.coli*'s survival. The effect of freezing temperature on microorganism reduction is: -5°C > -35°C >-15°C. The cells at exponential growth phase were more sensitive to the freeze/thaw process than the stationary growth phase cells. In the spraying freezing test, samples collected two days after spraying, the number of *E.coli* surviving dropped dramatically. *Bacillus megaterium* spores reduction with freeze/thaw process showed stronger tailing phenomenon after UV exposure than without freezing. At low chlorine concentration (2.0 mg/L), this process made spores more resistant than at higher concentration (6.0 mg/L). It appeared that the freeze/thaw process caused damage to the cell structure, but also decreased the water activity and clumped the spores. In spraying freezing test, there was no significant effect on the survival of spores.

Acknowledgments

The author wishes to express his sincere appreciation to Dr. Daniel W. Smith and Dr. Wa Gao for their guidance, encourage, patience and understanding throughout the experimental period. I also wish to thank Mrs. Maria Demeter for her help through my whole experimental work. Without their support, the completion of this project would not be possible.

The author is also indebted to Mr. Nick Chernuka, Mr. Garry Solonynko and Mr. Rodney Guest for their assistance in the installing and maintaining the experimental equipment and in growing the microorganisms.

I also want to thank Dr. Ming Chen and Mr. George D. Braybrook for their help in taking the SEM images of microorganisms.

Finally, I want to thank the academic and support staff of the Environmental Engineering and Science Program, for encouragement and practical help in my attainment of this degree.

Table of Contends

Chapter 1. Introduction 1

1.1. Overview 1

1.2. Microbe Selection 3

1.3. Objectives 4

Chapter 2. Literature Review 5

2.1. Physical Events Occurring in Freezing and Thawing 5

2.2. Impurity Separation by Freezing 8

2.3. Factors Affecting Microbial Survival 10

2.3.1. Type and Strain of Microorganism 10

2.3.2. Nutrition Status 12

2.3.3. Composition of Cooling and Freezing Medium 13

2.3.4. Growth Phase and the Rate of Growth 14

2.3.5. Rate of Cooling 15

2.3.6. Storage at Low Temperature After Freezing 19

2.3.7. Thawing 20

2.4. Freeze Injury and Death 21

2.5. Mechanisms of Freeze Damage 23

2.6. Application of Freeze/Thaw Process 24

Chapter 3. Reduction of *Escherichia coli* by Freeze/Thaw Process 28

3.1. General Biology of *Escherichia coli* 28

3.2. Experimental Materials and Methods 29

3.2.1 Materials preparation 29

3.2.2. Microbial Preparation 30

3.2.3. *Escherichia coli* Enumeration 31

3.2.4. Experimental Design 33

3.2.5. Experiments 36

3.2.6. Statistical Analysis 41

3.3. Results and Discussion 41

3.3.1. Fractional Factorial Experiment Design 41

3.3.2. Effect of Freezing on the Survival of *E.coli* at Different Growth Phase 44

3.3.3. Effect of Freezing Storage Time on *E.coli* Survival 47

3.3.4. The Effect of Freezing Cycles on *E.coli* Survival 56

3.3.5. Spraying Freezing Test for *E.coli* Reduction 60

3.4. Conclusion 64

Chapter 4. Reduction of the Spores of *Bacillus megaterium* by Freeze/Thaw Process 65

4.1. General Biology of *Bacillus spp.* 65

4.2. Experimental Materials and Methods 67

4.2.1 Materials Preparation 67

4.2.2. Preparation of *Bacillus megaterium* 68

4.2.3. *Bacillus megaterium* Enumeration 70

4.2.4. Experiments 71

4.3. Results and Discussion 74

4.3.1. The Effect of Freeze/Thaw Process on *Bacillus megaterium* 74

4.3.2. Spraying Freezing Test for *Bacillus megaterium* Reduction 78

4.3.3. Freezing Combined with UV Test for *Bacillus megaterium* Reduction 78

4.3.4. Chlorination for *Bacillus megaterium* Reduction 82

4.5. Conclusion 86

Chapter 5. Summaries and Recommendations 87

5.1. Summaries 87

5.2. Potential Use of the Freeze/thaw Process 88

5.3. Recommendations 89

References 90

Appendix A. Raw data of Experiment 99

Appendix B. Data Analysis for *E.coli* Reduction 133

Appendix C. Data Analysis for *Bacillus megaterium* Reduction 145

List of Tables

Table 3-1. 2^{5-1} Fractional Factorial Design....35

Table 3-2. Summary of Regression Statistics for m-FC Method....42

Table 3-3. Summary of Regression Statistics for Modified m-FC Method....42

Table 3-4. Univariate Analysis of Variance for Freezing Storage Test....49

Table 3-5. Average Injured *E.coli* Percent at Different Temperatures....50

Table 3-6. *E.coli* Reduction for the Freezing Cycles at Different Temperatures....56

Table 3-7. Univariate Analysis of Variance for Freezing Cycle Test....58

Table 3-8. Paired t-test for *E.coli* Reduction of Two Test Methods....60

Table 3-9. t-test for *E.coli* Reduction of Spraying and Batch Tests....61

Table 4-1. t-test for *Bacillus spp*. Reduction....74

Table 4-2. t-test for *Bacillus spp*. Reduction in Spraying Test....78

Table 4-3. UV Light for *Bacillus megaterium* Inactivation....79

Table 4-4. Chlorination for *Bacillus megaterium* Reduction....83

List of Figures

Figure 2-1. The Relationship of a_w to Temperature.......7

Figure 2-2. The Whole Eutectic Diagram of Water and a Compound X.......9

Figure 2-3. Effects of Temperature of Freezing on Viability of *E.coli*.......14

Figure 2-4. Effect of Cooling rate on the Survival of *E.coli* in Water or in Saline.......16

Figure 2-5. Comparative Effects of Cooling Rate on the Survival of Various.......16

Figure 2-6. Microbial Survival Ratio S (%) vs. Freezing Speed.......17

Figure 3-1. Schematic of Spraying Test Apparatus.......39

Figure 3-2. *E.coli* Reduction in Exponential Phase.......45

Figure 3-3. *E.coli* Reduction in Growth phase.......46

Figure 3-4. *E.coli* Reduction vs. Storage Time (m-FC Method).......47

Figure 3-5. *E.coli* Reduction vs. Storage Time (Modified m-FC Method).......48

Figure 3-6. Injured *E.coli* Percent During Storage.......50

Figure 3-7. *E.coli* Reduction vs. Freezing Cycles (m-FC Method).......57

Figure 3-8. *E.coli* Reduction vs. Freezing Cycles (Modified m-FC Method).......58

Figure 3-9. Percentage of Injured *E.coli* Cells vs. Freeze/Thaw Cycle.......59

Figure 3-10. *E.coli* Reduction vs. Accumulative Melt Ice Percent.......63

Figure 3-11. Comparison of *E.coli* Reduction for Ice Storage.......63

Figure 4-1. Schematic Illustration of Sporulation Stages in *Bacillus subtilus*.......66

Figure 4-2. Comparison of *Bacillus spp*. Reduction with or without Freezing.......80

Figure 4-3. Chlorination (2.0 mg Cl_2/L) for *Bacillus megaterium* Reduction.......84

Figure 4-4. Chlorination (4.0 mg Cl_2/L) for *Bacillus megaterium* Reduction.......84

Figure 4-5. Chlorination (6.0 mg Cl_2/L) for *Bacillus megaterium* Reduction.......85

List of Images

Image 3-1. Image of *E.coli* without Freezing Effect..52

Image 3-2. Image of *E.coli* After Freezing Effect..53

Image 3-3. Image of *E.coli* After Freezing Effect..54

Image 3-4. Image of *E.coli* in the Ice..55

Image 4-1. *Bacillus megaterium* (spore) Before Freezing..75

Image 4-2. *Bacillus megaterium* (spore) After Freezing..76

Image 4-3. *Bacillus megaterium* (spore) in the Ice..77

Chapter 1. Introduction

1.1. Overview

In Canada, the cold weather is always an important factor that should be carefully considered in water and wastewater treatment process design. Because of the long winter, the selection of an effective treatment method, which was restricted by technical and economical reasons, was a challenge to the water and wastewater engineers. Because low temperature would reduce the chemical and biological reaction rate, restrain the growth of microorganisms and also can increase the water viscosity that would affect the transfer of materials in the water.

In America, passage of Federal Water Pollution Control Act Amendments of 1972, as amended in 1977 and 1978 (Clean Water Act), stimulated substantial changes in wastewater treatment to achieve the principal objective of the Act, which was to make the nation's waters "fishable and swimmable" (Metcalf & Eddy, Inc., 2003). So how to efficiently remove the microorganisms, especially the pathogens in the wastewater, has become one of the major problems in wastewater treatment.

Using chlorine for microorganism reduction (MOR) had been extensively studied in North America since 1970's in response to the inclusion of a universal fecal coliform standard for wastewater discharges in the Clean Water Act of 1972 (Haas, 1986). The potentially adverse effects caused by the extensive use of chlorine as a MOR chemical was recognized by people. The search for effective alternative MOR chemicals or methods to chlorine has become a new task. Ultraviolet light, ozone, chlorine dioxide and even several other halogens have been evaluated and considered as alternatives to chlorine. But each of these MOR chemicals and methods has potential to form by-product and each is affected by the temperature. At the low temperature, the efficiency of microorganism reduction will decrease. So, how to utilize cold temperature to treat wastewater and remove pathogens has become an interesting topic for researchers working in cold regions.

Natural freezing technology, which can concentrate the impurities in the unfrozen liquid and produce purified ice crystals, could be one method that can provide a practical treatment alternative for wastewater treatment (Gao, 1998). There are many studies using freezing for industrial wastewater treatment and sludge conditioning. Martel (2000), Sanin *et al.* (1994) and Martel & Diener (1991) had found that many kinds of microorganisms could be reduced in numbers with freezing treatment.

It is known that some microorganisms have survived for long periods at subfreezing temperatures being recovered from remote polar locations. As an example, some scientists found viable microorganisms in the accreted ice above one of the subglacial lakes in Antarctica (Karl *et al.*, 1999). Whether and how do the microorganisms respond to the effect of freezing should be considered in the application of freezing and thawing technology. Previous studies had found that freezing negatively affected most types of pathogens, but many could survive (Parker and Martel, 2002). For example, Sanin *et al.* (1994) found frozen under −18 °C for 1 day, the overall reduction of fecal coliform was just 0.1 log-unit and Plaque forming units was 0.48 log unit.

In this study, we examined more closely the effects of low temperature, freezing and thawing on selected microorganisms and explored their survival. Cells damage in the frozen environment was closely examined.

1.2. Microbe Selection

In this study, a kind of gram-negative bacteria, which represented the most sensitive bacteria to the freeze/thaw process and spore, which represented the most resistant bacteria to the freeze/thaw process would be selected as the study microorganisms. *E.coli* is a gram-negative and nonspore-forming bacterium. It is a very important indicator in the water and wastewater treatment and it can be used as a measure of recent fecal contamination. Most strains of *E. coli* are harmless and live in the intestines of healthy humans and animals, but *E. coli* O157:H7 can cause of foodborne and waterborne illness (USEPA, 2004). The water outbreak case in Walkerton, Ontario were identified as the water contaminated by *E. coli* O157:H7 (Hrudey, S. and Hrudey, E., 2004).

Bacillus spp. are aerobic, endospore-forming, gram-positive bacteria. In suitable environment, they can form spore. *Bacillus subtilis* and *Bacillus megaterium* have been popular used in many microorganism reduction studies. Comparing with *Bacillus subtillus*, *Bacillus megaterium* tends to produce higher concentration of spores (Guest, 2004). So, in this project, these two kinds of bacteria were selected as the study microorganisms.

1.3. Objectives

The objectives of this study are:

- to investigate removal efficiency of microorganisms by freeze/thaw process;
- to determine which factors have significant effect on the microorganisms survival in the freeze/thaw process;

- to examine how the freezing temperature, ice storage time, freezing cycles and other factors such as: the growth phase of microorganisms, affect the microorganisms viability;
- to evaluate the different effects on the microorganisms between batch test and continuous spraying freezing treatment systems;
- to investigate how the freeze/thaw process affect microorganisms' structure and distribution within ice columns; and
- to investigate how the UV and chlorination process affect the survival of spores after freeze/thaw process.

Chapter 2. Literature Review

Freezing and thawing has been used as a process to preserve biological structure for a long time. The study of the effects of freezing on the survival of microorganisms probably can be dated back to 18th century (Calcott, 1978). Today, one of the major reasons for studying the effects of freezing on microorganisms is to minimize their survival and prevent their multiplication in materials, such as foods, water or ice, which may relate to human health. It appears that many factors influence the survival of microbes during freezing and thawing. Associated with death are the formation of the ice crystals and the concentration of solutes both internal and external to the cell (Calcott, 1978). So, the freezing process changes the distribution of physical materials in the solid-liquid phase, and also change the structure of organisms.

2.1. Physical Events Occurring in Freezing and Thawing

When an aqueous solution is cooled from temperatures above 0°C, the solubility of the solutes may change slightly, but generally, with the exception of saturated solutions, the solution remains liquid until reaching its freezing point at some temperature below 0°C. The precise freezing point is dependent on the concentration and nature of the solutes present. When the ice begins to form, the dissolved solutes are concentrated in the remaining liquid. As the temperature is further reduced and more water is converted into ice, solute concentration rises more and more in the unfrozen water portion with corresponding decreases in freezing point and water activity (a_w) (Ingram and Mackey, 1976).

Water activity (a_w): is the ratio of the water vapor pressure in any food system to the water vapor pressure of pure water at the same temperature, defined as $a_w = p/p_0$ where p is the partial pressure of water above the material and p_0 is the partial pressure of pure water at the same temperature (Patti Wilson, 2004). It should be noted that water activity is a colligative property, that is to say it depends on the number of molecules or ions present in solution, rater than their size. Thus a compound like sodium chloride, which dissociates into two ions in solution, is more effective at reducing the water activity than a compound. In physical chemistry, it prefers to work with the chemical potential of water. The relationship between the water potential and the water activity can be described as: $\psi = RT \cdot \ln a_w / V_m$, where, R: the gas constant, V_m: the molar volume of water, T: temperature and a_w: water activity (Adams and Moss, 2000). Water potential may contain both an osmotic component, associated with the effect of solution, and a matric component, associated with the interaction of water molecules with surfaces, which can be clearly demonstrated by the rise of water in a capillary tube. A parameter related to water activity is osmotic pressure, which can be thought of as the force per unit area required to stop the net flow of water molecules from a region of high to one of low water activity. With the reduction of water activity, the osmotic pressure will increase. Cytoplasm is an aqueous solution and so must have a lower water activity and higher osmotic pressure than pure water; thus a micro-organism in an environment of pure water will experience a net flow of water molecules into the cytoplasm (Adams and Moss, 2000).

So, water activity describes the continuum of energy states of the water in a system and reflects a combination of water-solute and water-surface interactions plus capillary forces. One of the major applications of water activity concerns the control of microbial growth. Most pathogenic bacteria in food can be stopped by water activity of around a_w 0.90, but to stop yeasts and molds it is necessary to lower activity to as little as a_w 0.7 to 0.75 (Food Science Australia, 2004).

The relationship between cooling rate temperature and water activity is represented in Figure 2-1. The relationship of a_w to temperature for ice and water mixtures is represented by line AB. The effect of lowering temperature on the a_w of a high solute liquid is shown by line CDB where the freezing point is depressed to D.

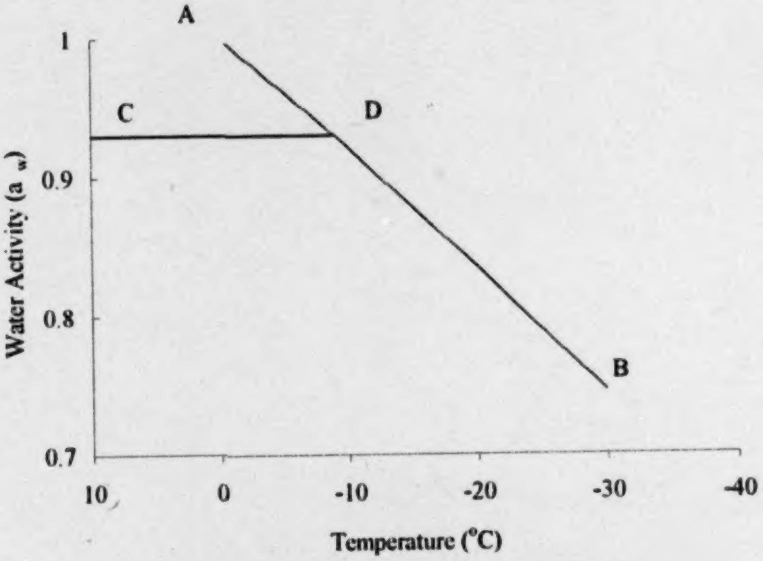


Figure 2-1. The Relationship of a_w to Temperature
(After Ingram and Mackey, 1976)

The concentration of solutes continues to increase as the temperature is lowered, until the eutectic point (D) is reached and the remaining solution is then solidified. The lowest temperature at which the solution remains liquid is referred to as the eutectic temperature. In the case of NaCl, the eutectic temperature is -21.8 °C, and the concentration attained before solidification is approximately 5 M (Meryman, 1966).

The size of ice crystals formed on freezing is primarily dependent on freezing rate. If solutions are frozen at ultra-rapid rates (several thousand degree per minute), few or no ice crystals can be observed but they can be detected by warming the samples to, for example, -30°C and allowing crystal growth to visible proportions. The slow freezing rates (typically 1°C/minute) can produce large crystals which can exceed the dimensions of microbial cells (Robinson, 1985).

2.2. Impurity Separation by Freezing

The principle of freeze separation is based on the fact: when ice is crystallized from an aqueous solution, the ice crystal is essentially built up by pure water, leaving the solutes in the remaining liquid phase. Several kinds of diagrams exist to present a binary mixture and the eutectic form is most often encountered for water and a soluble compound, because, theoretically, they only required a single step to remove compounds (Lorain *et al.*, 2001). Freezing of a binary solution of water and a compound X is presented in Figure 2-2.

The path AB represents the cooling of solution to its solidification temperature. Freezing of pure water starts at point B and continues until point P, which represents the system at equilibrium for a given temperature. Point P gives the proportions and concentrations of the phases, as shown in Figure 2-2: SP/SQ = liquid proportion and PQ/SQ = solid proportion. The concentration of X in liquid will increase from X_a to X_q and the liquid phase is always keeping 100% of water. The eutectic temperature is the boundary of the freezing process. Below it, the co-precipitation of water and boundary of the freezing process. Below it, the co-precipitation of water and compound X occur at the same time. So, the idea of using freezing for water or wastewater treatment is that pure water ice will be produced while the pollutant is concentrated in the remaining liquid.

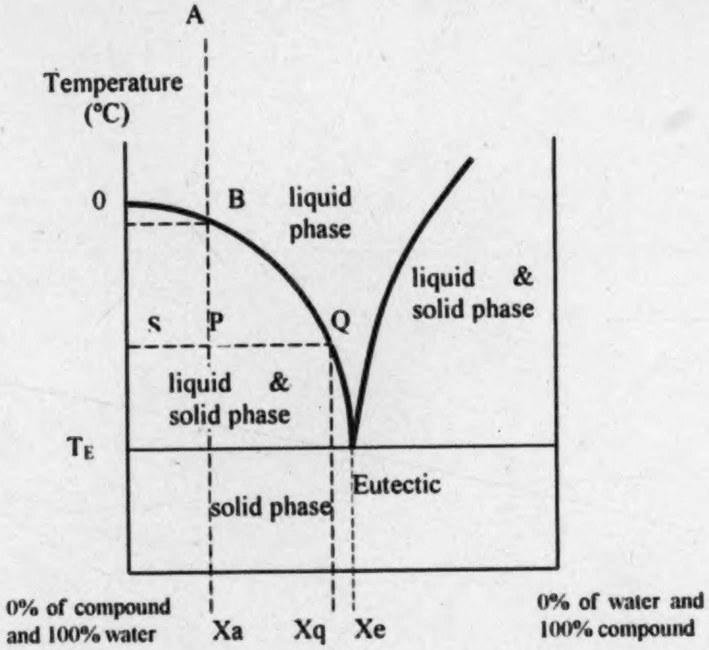

A
Temperature (°C)
0
B
liquid phase
liquid & solid phase
S
P
Q
liquid & solid phase
T_E
Eutectic
solid phase
0% of compound and 100% water
Xa
Xq
Xe
0% of water and 100% compound

Figure 2-2. The Whole Eutectic Diagram of Water and a Compound X
(After: Lorain *et al.*, 2001)

In complex water or wastewater, because there is not only one solute but numerous different compounds, the situation is more difficult to predict. The phase diagram of such a wastewater changes with the addition of each new compound.

Normally, water and wastewater always contain both soluble and non-soluble compounds. For most dissolved salts, the solubility is higher in the liquid phase than in the solid phase. Freezing of a salty solution leads to the enrichment of the liquid phase and to the purification of the solid one. Because the structure of the ice crystal has great regularity and symmetry, therefore, the structure cannot accommodate other atoms or molecules without very severe local strain, practically every solute in the water is rejected by the advancing surface of a growing ice crystal (Chalmers, 1964).

At sufficiently small growth velocities, particles of nearly all materials are rejected by a moving solid liquid interface, therefore, the non-soluble compounds are pushed ahead into the liquid (Halde, 1980). The particle is acted on by forces such as gravity and viscous drag promoting contact. If the particle is an ideal sphere, it will be in single contact with the ideal curved solid-liquid interface. The larger the particle diameter is, the lower is the critical velocity for particle trapping. An increase in contact area at constant particle size causes increased migration (Halde, 1980).

When microbial cells are suspended in the aqueous solution, they behave like solute molecules and are concentrated in the unfrozen portion of the solution by the growing of ice crystals. Because these cells are partitioned into an increasingly concentrated solution, they are exposed to the forces of this environment and to the consequences of localized ice crystal growth (Calcott, 1978).

2.3. Factors Affecting Microbial Survival

2.3.1. Type and Strain of Microorganism

Many psychrophilic microorganisms, which can thrive at relatively low temperatures are capable of growth at sub-zero temperatures. It appears that growth at -5°C to -7°C can be observed, although only rarely at temperatures below -10°C. For example, *Bacillus psychrophilius* can grow at -5°C to -7°C with a generation time of 204 hours (Davies and Obafemi, 1985). Cold shock, freezing, storage at low or subzero temperatures can injure or be lethal to bacteria. Cold shock is caused by sudden chilling without freezing. Early studies have shown that cold shock can damage the cytoplasmic (inner) membrane and DNA of bacteria, and the outer membrane of gram-negative bacteria (Macleod and Calcott 1976, Mackey, 1984). Differing sensitivity to 'cold shock', on the other hand, maybe a more significant factor as it has long been considered that thermophiles and mesophiles were more susceptible to low temperature shock than psychrophiles (Jay, 1978). However, Ingram and Mackey (1976) pointed out that cold shock effects depend more on the magnitude of any temperature drop rather than on the actual temperature at which the cells were grown; this has been demonstrated in psychrophiles as well as mesophiles, and in some *Gram-positive* bacteria, *streptomycetes* and *Gram-negative* bacteria. Factors that affect the sensitivity of cells to cold shock include age, composition of medium in which cell are chilled, cells number and rate of cooling (Parker *et al.*, 2000 and Mackey, 1984).

Freezing and thawing could damage the cytoplasmic membrane, cell wall and DNA (Macleod and Calcott, 1976, Ray, 1989). When the cytoplasmic membrane is damaged, low molecular weight materials (such as potassium and magnesium cations, inorganic phosphate and amino acids) are lost from the cell, and small molecular compounds, such as toxic metals can penetrate into the cell (Macleod and Calcott, 1976). The death or injury of bacteria can be attributed to one or both of these processes. However, depending on the species and the surrounding medium, many cells injured by these processes can self-repair (Davies and Obafemi, 1985).

Microorganisms can be grouped according to differences in their inherent responses to freeze–thaw stress by using the categories of Mazur (1966), that is:

a) survive all conditions of freezing and thawing;

b) resist the immediate effects of freezing but are sensitive to frozen storage;

c) sensitive to both immediate and storage effects of freeze under some conditions; and

d) sensitive to freezing and frozen storage under all conditions.

The first category includes most spores. Bacterial endospores are extremely resistant to freezing and to storage at sub-zero temperatures, with survival levels exceeding 90%. This can be attributed to the relatively dehydrated state of the spore protoplast with much of its water bound in an unfreezable state within the expanded cortex (Davies and Obafemi, 1985). Some gram-positive *staphylococci, micrococci* and *streptococci* are relatively resistant, with survival exceeding 50% (Mazur, 1966).

Organisms which are very sensitive to the effects of freezing include the free amoebae, ciliated protozoa and nematodes. The cooling rates are critical. The majority of micro-organisms are in categories b) and c). Generally, most Gram-positive organisms including *Bacillus, Clostridium, Corynebacterium, Lactobacterium, micrococcus, Staphylococcus* and *streptococcus*, together with some yeasts, are relatively resistive to freezing, though some are very sensitive to the frozen storage (Robinson, 1985). Gram-negative organisms, such as *Escherichia spp., Salmonella spp., Serratia spp., Pseudomonas Acinetobacter-Moraxella* and *Vibrio spp.*, are considerably more sensitive to both freezing and frozen storage (Ingram and Mackey, 1976), and their survival is dependent on cooling velocity, temperature, cell concentration, storage time and thawing conditions (Mazur, 1966).

2.3.2. Nutrition Status

The nutrition status of microorganisms can influence their resistance to freezing.

As Gilliland and Speck (1974) noted that the strains of lactic *Streptococci* showed cryosurvival properties dependent on the level of various polymers in the cell and it is possible to manipulate the cryosuvival by altering those growth conditions which promote the synthesis of protective polymers. Calcott and Macleod (1974) have investigated this factor by growing cells of *Escherichia coli* under different carbon or nitrogen limited conditions in continuous culture. They found that nitrogen-limited cells accumulated higher carbohydrate contents and were more resistant to freezing, and this observation suggested that polyglucose and glycogen-like reserve materials could be cryoprotective by strengthening the cell envelope or outer membrane. In cultures grown in highly aerobic conditions, a high trehalose content, which had been recognized as a particularly effective cryoprotective carbohydrate and its effect had been attributed to membrane stabilization, was correlated with resistance to freezing (Lund, 2000). Gelinas *et al.* (1989) found that strong aeration in addition to fed-batch culture gave the highest resistance with up to 92% survival of a baker's yeast, *Saccharomyces cerevisiae*, which was frozen to –50°C for 10 minutes in water.

2.3.3. Composition of Cooling and Freezing Medium

The survival of some Gram-negative bacteria is lower in NaCl solution than in water (Lund *et al.*, 2000). Calcott and Macleod (1974) had found that *E.coli*, frozen at temperatures below -20°C in saline, showed much lower viability than those frozen in water (Figure 2-3). In contrast to *E.coli*, *Streptococcus faecalis* was resistant to the presence of NaCl during freezing (Calcott *et al.*, 1976).

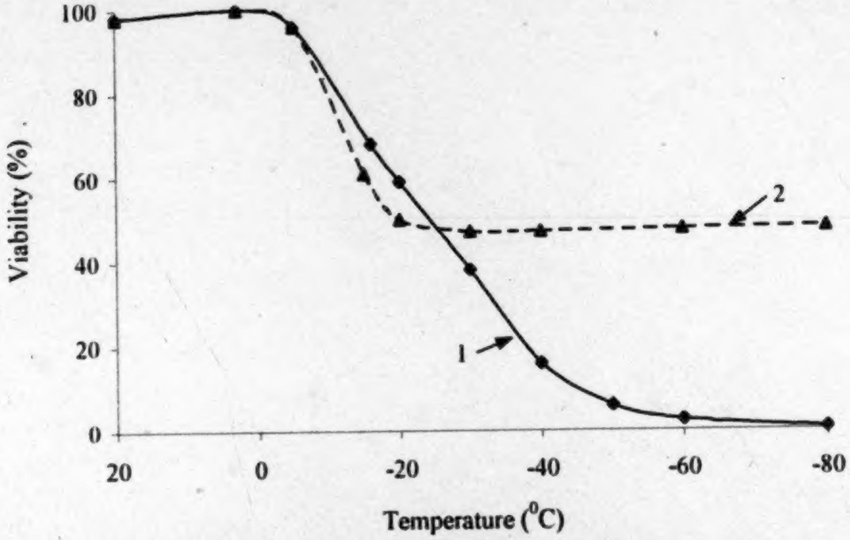


Figure 2-3. Effects of Temperature of Freezing in Distilled Water (curve 2) and in 0.85% Saline (curve 1) on Viability of *E.coli*.
(After: Calcott and Macleod, 1974)

An acidic medium may reduce the survival of bacteria. Adjusting pH of trypticase soy broth from 7.3 to 3.8, during freezing to -30°C, *Staphylococcus aureus* decreased survival by eight-fold and a high proportion of survivors were sublethally injured (Minor and Marth, 1972).

2.3.4. Growth Phase and the Rate of Growth

The response of cells to freeze/thaw is different at different growth phases. Exponential phase cells are much more sensitive to freezing and thawing than stationary phase cells. The mechanisms of growth-phase-related changes in cryosensitivity have not been subjected to definitive study. Ray and Speck (1973) suggested that the differences merely reflect differences in physiological activity and hence vulnerability between the two states of growth. However, Davies (1970) found that the sensitive of *E.coli* to freezing, at different phases of growth, was dependent on freezing rate. At some rates, exponential phase cells survived better than stationary phase ones, while at other rates the opposite was true. This work was extended by Calcott and Macleod (1974) using chemostat populations of *E.coli* grown at various rates. At low cooling rates, they found the cells were more sensitive to freezing stress as their growth rates increased, but at rates between 10 and 100°C/min, the relationships were reversed.

2.3.5. Rate of Cooling

Most cell types, prokaryotes or eukaryotes have an optimum cooling rate for survival that varies, depending on the water permeability of the membrane and on the surface-to-volume ratio of the cell (Mackey, 1984). For many bacterial species, maximum survival occurs at cooling rates between 6 and 11°C/min (Mazur, 1966, Macleod and Calcott, 1976 and Mackey, 1984). Figure 2-4 shows the effect of cooling rate on the survival of *E.coli* in water and in a saline solution. The cells have an optimum survival in the slow cooling rate range, but as the rate of cooling is increased or decreased, survival is reduced.

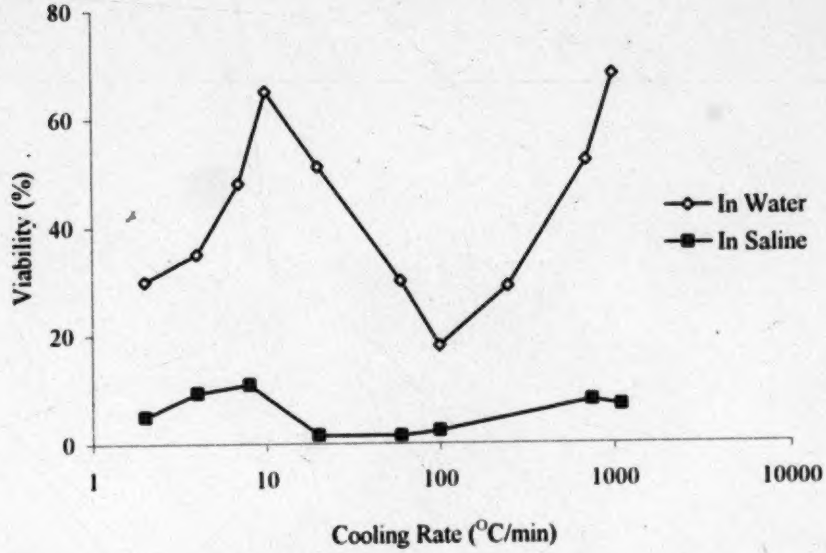


Figure 2-4. Effect of Cooling Rate on the Survival of *E.coli* in Water or in Saline. (After: Robinson, 1985)

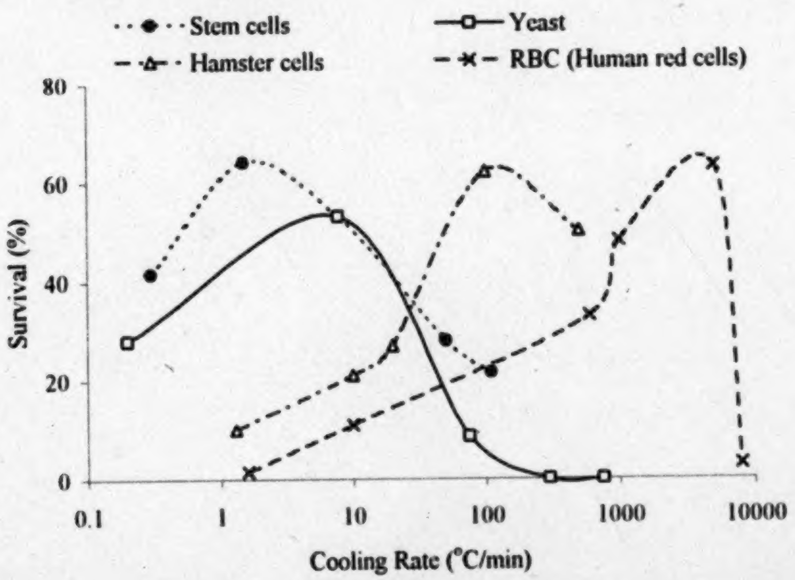


Figure 2-5. Comparative Effects of Cooling Rate on the Survival of Various Cells. (After: Calcott, 1978)

Figure 2-5 compares the effect of cooling rate on the survival of various cells (Calcott, 1978). The optimum cooling rates for cell survival are varied for the type of cells.

As studied by Chu *et al.* (1999), the survival ratio of the microbes (total coliforms, TC and heterotrophic plate count, HPC) increased as the freezing speed increased. Figure 2-6 presents the results by Chu *et al.* (1999). In this study, the total coliforms were observed having higher survival ratio in the same cooling speed. Heterotrophic bacteria such as *Pseudomonas spp.*, *Alcaligenes spp.*, *Paracoccus spp.*, *Flavobacterium spp.* and *Coryneform spp.* were not able to form an endospores or capsules to resist freeze/thaw treatment; meanwhile, total coliforms such as *Escherichia spp.* and *Klebsiella spp.* were able to form capsules.

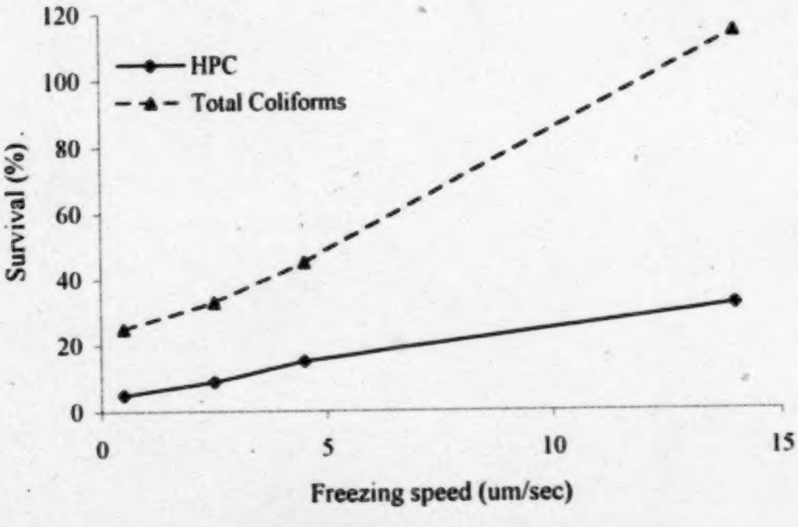


Figure 2-6. Microbial Survival Ratio S (%) vs. Freezing Speed
(After: Chu *et al.*, 1999)

Sanin *et al.* (1994) found the rate of freezing greatly affected the reduction in fecal coliform and somewhat enhanced the removal of *Salmonella spp.* At -25°C, the removal rate of fecal coliform was 1.10 log unit and *Salmonella spp.* was 0.70 log unit. At -7°C, the removal rate of fecal coliform was near to zero and *Salmonella spp.* was just 0.26 log unit. Virus removal, as measured by plaque forming units, was not affected by the rate of freezing. In the study, high reduction of fecal coliforms was observed while the fecal *streptococci* presented more resistant to freezing than fecal coliforms and *Salmonella spp.* The authors also observed that the freezing/thaw sludge conditioning was very effective in reducing *Cryptosporidium parvum*. After freezing, no viable sporozoites were observed and the reduction was much higher than 1.0 log.

The existence of an optimum cooling rate suggests that there are at least two mechanisms of damage which are oppositely affected by rate of cooling. Mazur (1966) proposed that at very slow cooling rate (<10°C/min), below the optimum for survival, extracellular freezing takes place and cell damage is mainly caused by exposure to increasing concentrations of solutes, thereby causing the cell to dehydrate. Solute concentrations inside and outside the cell then reached levels that caused denaturation of proteins and breakdown of membrane. At higher cooling rates (>10°C/min), the temperature drops at a faster rate than water can flow through membrane and then causes the nucleation of ice in intracellular water. At ultra-rapid cooling rates (>100°C/min), ice crystal growth is retarded or prevented and makes the intracellular ice crystals so small that the survival of microorganisms again gets higher. If these cells are warmed slowly, small ice crystals may grow and then cause cell damage.

2.3.6. Storage at Low Temperature After Freezing

Several studies have shown that in addition to the death of cells on initial freezing, there is usually further death during frozen storage. Mazur (1966) indicated that the longer the period of holding at sub-zero temperatures, the lower the survival. Usually, the rate of death of organisms during freezing storage is greatest when held at or just below freezing temperatures. As the holding temperature is lowered, the death rate reduces, and below –60 °C the rate of death is usually very low (Macleod and Calcott, 1976). After freezing, death is initially fairly rapid, particularly at –2°C to –5°C, and then gradually slows in the later stage storage. According to Mazur (1966), the death rates are low or zero when storage temperatures was -70°C or below. The death rate depends on the species, the storage temperature, the nature of the freezing medium and in some cases the cell concentration (Mazur, 1966, Macleod and Calcott, 1976). The decline in viability is probably because of continued exposure to concentrated solutes, and thus represents an extension of the immediate stresses associated with freezing at low cooling rates (Ingram and Mackey, 1976).

In the study of Sanin *et al.* (1994), freezing time and temperature combination was found important for the removal of plaque forming units. Shorter times are sufficient for pathogen removal if freezing temperature is kept low.

The response of bacteria to frozen storage was different. Mackey (1984) indicated that at -20°C and a storage time for a few weeks, fecal *Streptococci* and *Staphylococcus aureus* survived well under most conditions, whereas *Vibrio parahaemolyticus, Yersinia enterocolotica* and vegetative cells of *Clostridium perfringens* declined in numbers by as much as 10^2 to 10^5, and other organisms such as *Salmonella* species and *E.coli* were of intermediate resistance, with their survival highly dependent upon the composition of the frozen medium.

McCarron (1965) studied the survival of six bacteria, which included three gram-negative rods (*E.coli*, *Aerobacter aerogenes*, and *Serratia marcescens*), two gram-positive cocci (*Micrococcus roseus* and *Sarcina lutea*) and spores of *Bacillus subtillis*, in ice between -2 °C and -20°C. He found that more than 90% of the bacteria were inactivated in the first few days and the remaining cells could persist for several months. The spores of *Bacillus subtilis* were most resistant to freezing and *E.coli* succumbed more rapidly than the others during storage. In general, the resistance of cells to frozen storage are: spores > gram-positive bacteria > gram-negative bacteria.

2.3.7. Thawing

Calcott (1978) pointed out that the rate of thawing generally has little effect on the survival of microbial cells frozen at a cooling rate < 100°C/min. At rapid cooling rates, subsequent survival is considerably greater at rapid warming rates than slow thawing. This can be explained as a consequence of ice crystal growth during slow thawing which is prevented or minimized in conditions of rapid thaw.

In contrast, Obafemi (1983) has shown that the thawing of exponential phase cells of *S.typhimurium* at 4 °C for 80 minutes is more lethal than thawing at 40°C for 13 minutes, with an attendant increase in the deoxycholate sensitive proportion of the survivors at 4°C. This effect was not observed for stationary phase cells (Robinson, 1985).

2.4. Freeze Injury and Death

Many bacteria that are believed to be killed by freezing, frozen storage, or thawing (especially Gram-negative bacteria) are actually only injured as a result of sublethal physiological and/or structure change (Lund *et al.*, 2000). A bacterium is termed injured if it can form colonies on nonselective medium but not on medium that contain selective agents (Parker and Martel, 2002). The exact mechanisms by which freezing causes viability loss in microbial cells are not fully understood (Ray, 1983), although many studies have been conducted on the nature and sites of freeze-injury. The ways by which freeze-injury has been demonstrated include: loss of viability; leakage of cellular materials; increased sensitivity; increased nutritional need (Davies and Obafemi, 1985).

Some studies had shown that there was a correlation between the loss of viability and the quantity of cellular material leakage. Lindeberg and Lode (1963) found that death in frozen *E.coli* was proportional to the amount of nucleic acid lost through leakage. Calcott and Macleod (1975) demonstrated a release of UV-absorbing material which was inversely proportional to the survival of frozen cells of *E.coli*. It appeared that leakage was determined by the cooling rate. Other materials were found to have leaked into the suspending medium of frozen cells, which included: biologically active peptides, cellular proteins, DNA, amino acids, etc. (Davies and Obafemi, 1985).

After freezing, some Gram-negative bacteria demonstrated an increased sensitivity to surface active agents and other compounds, which could be tolerated by healthy cells and this had facilitated the distinction between the structurally injured and the unharmed cells in a frozen population (Ray and Speck, 1973). After freezing, the injured *E.coli* cells failed to multiply and develop colonies in the presence of bile salts or deoxycholate on the media (Ray and Speck, 1973). It was reported that *E.coli* had developed sensitivity to actinomycin D, which normally did not penetrate the Gram-negative cells (Bretz and Kocka, 1967). From these studies, it could led to conclude that the barriers that normally protect cells became impaired by freezing and then allowed the compounds to penetrate into the cells to bring about their inhibitory effects (Ray and Speck, 1973).

The other phenomenon of frozen injured cells is the increased nutritional needs. The failure to form colonies on minimal medium was attributed to the inability function as required for growth and multiplication. Ray and Speck (1972) found if given suitable nutrients, *E.coli* and *Salmonella anatum* could repair their injured structure from freezing. They also found that the repair of metabolically injured *E.coli* had shown to be aided by the addition of low molecular weight peptides and that adenosine triphosphate (ATP) synthesis was required for repair.

Previous studies had proven that the freezing/thawing process could damage the cell membrane, cell wall, nucleic acids and proteins (Davies and Obafemi, 1985). The membrane damage with associated permeability impairment, resulted not only in the loss of cellular material through leakage, but also the penetration by injurious substances from environment (Macleod, 1967). Membrane damage had been demonstrated by: increased salt sensitivity; increased sensitivity to EDTA; increased sensitivity to lysozyme; release of periplasmic enzymes and alkaline phosphatase (Davies and Obafemi, 1985). The studies for *E.coli* freezing had shown that the type of membrane damage is freezing-rate dependent. With a slow freezing rate (3 to 10°C/min), the damage was mainly caused by damage to the membrane. The rapid freezing (200°C/min), the damage was caused by damage to both the cytoplasmic membrane and the out membrane (Robinson, 1985).

In some Gram-negative bacteria the possession of the lipopolysaccharide layer (LPS) is considered a protection against several surface active and other injurious compounds. Evidence has been presented by Ray *et al.* (1976) that the LPS layer in the outer wall is damaged in cells of *E.coli* after freezing/thawing. Damage to this layer permitted lysozyme to enter the cell wall and dedrolyze the peptidoglycan.

Morichi (1969) suggested that the freezing might have interfered with the binding of ribosomes and thus allowed the degradation of the disrupted ribosomes. Gabis (1970) and Morichi (1969) had found the release of RNA from frozen cells and Calcotta and Macleod (1975) found UV-absorbing material released from frozen cells. The freezing process might have activated some latent proteolutic enzymes, therefore bringing about an increased breakdown of proteins. Gabis (1970) observed that large quantities of free amino acids were present in the freezing menstruum of frozen *E.coli* cells. He suggested that the presence of large amounts of basic amino acids might indicate that the ribosomal proteins had been hydrolysed in the frozen cells.

2.5. Mechanisms of Freeze Damage

The two factor hypothesis consolidated by Mazur (1966) predicted that death of cells as a result of freezing and thawing was due to two factors, one factor caused lethality at low cooling rates and other at higher rates. The presence of the optimum rate of cooling for a particular cell type was taken to be that when the sum of damage was minimal. It had been convenient to regard that intercellular ice causing damage in rapidly frozen cells and solute concentration affecting cells at low cooling rates.

When a cell is frozen, the temperature drop initially results in supercooling of the medium followed by ice crystal formation. The intracellular water remains unfrozen and supercooled until the temperature drops down to between -10°C and -15°C, above which only extracellular ice crystallization is expected to occur (Davies and Obafemi, 1985). The extracellular ice formation lowers the vapor pressure of the medium and the cell reacts to re-establish osmotic equilibrium either by freezing cellular water inside the cell, to reduce its 'availability', or by losing it and freezing extracellularly (Robinson, 1985). At a low cooling rate with high membrane permeability and high surface-volume ratios, ice forms extracellularly and cells dehydrate and shrink. Under these conditions, lethal effects result from high solute concentration inside the cells. At high cooling rates with low membrane permeability and low surface-volume ratios external ice grows through the water-filled pores of the membranes to nucleate the internal, supercooled cellular water which freezes intracellularly. Silvares *et al.* (1975) pointed out that the response of the cell during freezing was determined by a competition between mass and heat transfer. At low freezing rates, mass transfer predominated and a substantial portion of extracellular ice formed. At high freezing rates, heat transfer dominated as the temperature was reduced at a faster rate than the water can flow through the cell membrane. This resulted in ice nucleation in the intracellular water.

2.6. Application of Freeze/Thaw Process

Freezing is the most successful technique for long-term preservation of food since nutrient content is largely retained. At low temperatures, no microbial growth is possible (below 10°C). But freezing will not render an unsafe product safe- its microbial lethality is limited and preformed will persist (Adams and Moss, 2000). The survival rates of microorganisms after food freezing will depend on many factors, the nature of the food material and the compositions of freezing, but have been variously recorded as between 5 to 70%. Bacterial spores are virtually unaffected by freezing, most gram-positive bacteria are relatively resistant and gram-negatives show the greatest sensitivity. Food materials often act as cryoprotectants for bacteria so that bacterial pathogens may survive for long periods in frozen state. In one extreme example, *Salmonella* has been successfully isolated from ice cream stored at –23°C for 7 years (Adams and Moss, 2000).

The freeze/thaw process can also be used as an important method for bacteria preservation. The bacteria were stored at liquid nitrogen temperatures (-80°C) has been found to be the best method of maintaining ATCC bacterial strains for extended periods (over 99% have survived). Cell viability is increased as the storage temperature is decreased. When needed, bacteria can be revived by rapid thaw in a 37°C water bath, inoculate all contents into fresh medium and incubate under appropriate conditions (ATCC, 2004).

The freeze concentration technique is well known in chemistry for concentrating organic compounds. Shapiro (1961) first applied freezing as a laboratory method to concentrate organic compound and Baker (1967) used the freeze concentration as a pre-analytical method to concentrate trace organic compounds in order to increase analytical device efficiency.

Recently, freeze/thaw process was used to treat wastewater and for sludge conditioning. In 1986, Partyka (1986) presented a device, which worked at the triple point of water where ice was in balance with its vapor. A vacuum pump was used to draw the pure vapor up to a condenser. Industrial wastewater was successfully treated by using Partyka' device : metals was reduced to below 0.1 mg/L, and color, total dissolved solids, phosphates, chlorine and organics also had been removed by this process. Delta Engineering (Ottawa, Canada) has developed a patented process, called Snowfluent, which used secondary wastewater for making snow. According to the manufacturer, many of the contaminants could be concentrated and removed, such as total nitrogen (TKN), total phosphorus (TP) and BOD (Delta Engineering, 2001). Gao (1998) studied using spraying freezing to treat industrial wastewater. The unfrozen water generated in the spray freezing process could carry away more than 50% of impurities (indicated by TOC, COD, Color, Cl^- and SO_4^{2-}). Rabinowitz *et al.* (1988) reported that snowmaking reduced total coliforms and fecal coliforms concentration by 50%. Parker *et al.* (2000) used the freeze/thaw process for snowmaking to treat secondary wastewater. They found that gram-negative coliforms were the most negatively affected by this process, with losses of two and three orders of magnitude for fecal coliforms and total coliforms, respectively. Fecal *streptococci* were less adversely affected, with a loss of less than 72%.

Freeze/thaw conditioning prior to sludge dewatering is becoming increasingly popular because of the improvements in the design and efficiency of the facilities (Martel, 2000). The best application of freeze/thaw conditioning was on alum sludge. Martel and Diener (1991) observed that freeze/thaw conditioning dramatically converts alum sludge from fine particle suspension to a mixture of clear water and granular particles. They achieved a 96% reduction in volume. For activated sludge, after a freeze/thaw treatment, the bound water content of sludge cake, the floc volume and the sludge compressibility decreased, and the sludge filterability increased (Ormeci and Vesilind, 2001). Sanin *et al.* (1994) studied using freeze/thaw sludge conditioning for pathogen reduction. They found, for aerobically digested sludge frozen at –25°C and storage of 7 days, the reduction rates were high, fecal coliforms: 1.90 log units, *Salmonella:* 0.54 log, plaque forming units for virus: 0.80 log and protozoa (*Cryptosporidium parvvum* oocysts): >8.0 log units.

The other advantage of this technique was the simplicity of a physical process because no chemical compounds were added. Freeze concentration is suitable for all soluble pollutants, even the most toxic and for a large range of concentrations (Lorain *et al.*, 2001).

Chapter 3. Reduction of *Escherichia coli* by Freeze/Thaw Process

3.1. General Biology of *Escherichia coli*

Escherichia coli is a gram-negative, facultative anaerobic, nonspore-forming, motile, rod-shaped bacterium propelled by long, rapidly rotating flagella. It is the most common member of the genus *Escherichia*, named for Theodor Escherich, a German physician, who first isolated *E.coli* in 1884 (Columbia University Press, 2001). *E.coli* strains are defined mainly by their antigenic composition. Of taxonomic relevance are over 170 different serological types of lipopolysaccharide antigens (O antigens) and 80 types of capsular (K antigens). Other properties that are used to define individual strains are H antigens (flagellar proteins), F antigens (fimbrial proteins), and phage and colocin sensitivity (Schaechter, 2000).

E.coli is the most abundant facultative anaerobe in the feces and the colon of normal humans and many mammals. It is commonly present in concentrations of 10^7 to 10^8 live organisms per gram of feces. *E.coli* cells are periodically deposited from their intestinal residence into soils and waters. It has been believed that they don't survive for an extended number of days outside a host and could be cultured only for a few days after their introduction. For this reason, their presence has been taken as a measure of recent fecal contamination, and the coliform count of the drinking water supply or swimming facilities is still a common indicator of microbiological water purity (Schaechter, 2000).

E.coli is a chemoheterotroph that can grow on a large number of sugars or amino acids provided individually or in mixtures. The growth of strains is inhibited by the presence of single amino acids, such as serine, valine, or cysteine. *E.coli* can grow at temperatures between 8°C and 48°C, depending on the strain and the nutrient medium. Its optimum growth temperature is 39°C. The pH range for growth is between pH 6.0 and 8.0, although some growth is possible at about pH 1.0 (Schaechter, 2000).

In this section, *E.coli* (ATCC 15597) was selected as the study microorganism to investigate how did the *E.coli* response to the effect of freeze/thaw process, which factors had significant effect on the *E.coli* survival and how did the freeze/thaw process affect the cell structure and distribution within the ice phase.

3.2. Experimental Materials and Methods

3.2.1 Materials preparation

3.2.1.1. Buffer Solution

Batch reactor experiments were carried out using phosphate buffers. Buffers at pH 6.9 using 0.05 M phosphate concentration were prepared by dissolving 2.24 g/L disodium hydrogen orthophosphate and 4.76 g/L potassium dihydrogn orthophosphate (Fisher Scientific, Nepean, Ont.) in distilled water and autoclaved at 121°C for 15 minutes. 1.0 N NaOH was used to adjust the pH. The buffer solution was then stored at 5 °C.

3.2.1.2. Peptone Solution

1.0 mg Bacto™ Peptone Water powder (Difco, MD) was dissolved in 1.0 L distilled water. Using an automatic pipetting machine (Scientific Equipment Products, M.D.) to distribute 90 mL peptone solution into bottles and then autoclaved at 121°C for 15 minutes.

3.2.1.3. m-FC and Modified m-FC Agar

m-FC and Modified m-FC agar were prepared according to the manufacturer's instructions. 52 g/L m-FC agar (Fisher Scientific, Itasca, IL) was dissolved in distilled water, mixed and heated to boiling. 10 mL 0.01% rosolic acid was then added in the agar and heated to boiling. For the modified m-FC agar, the rosolic acid was not added. Using 1.0 N NaOH adjust pH to 7.2 to 7.4. Agar was poured into 47 mm Petri dishes in the laminar flow hood. After solidification, the prepared dishes were stored at 5°C in a refrigerator. The m-FC is also called selective medium and the Mm-FC, non-selective medium.

3.2.1.4. *E.coli* Grow Media

The *E.coli* growth media was prepared by dissolving 2.0 g/L Difco Nutrient Broth (Fisher Scientific, Itasca, IL) in distilled water, heating and mixing to boiling and then autoclaved at 121°C for 15 minutes.

3.2.1.5. Preparation of the Experimental Materials

All of the materials used in the experiments, including the plastic and glass beakers, were thoroughly cleaned in a dishwasher using Sparkleen soap (Fisher, Pittsburgh, PA) and then autoclave at 121°C for 10 minutes.

3.2.2. Microbial Preparation

E.coli ATCC strain 15597 (American Type Culture Collection, Rockville, MD) was used as the test microorganism. Difco nutrient broth (Fisher Scientific, Itasca, IL) was used as growth culture.

3.2.2.1. Nutrient Agar Preparation

4.0 g nutrient broth and 7.5 g granulated agar (Fisher Scientific, Itasca, IL) were dissolved in 500 mL distilled (DI) water and heated to boiling, and then put in an autoclaver sterilizered for 10 minutes at 121°C. After that, the sterilized agar was poured into the Petri dishes (100 × 15 mm) and stored in a fridge at 5°C.

3.2.2.2. *E.coli* Growth

Inoculate with *E.coli* from a nutrient agar slant previously cultured with the ATCC *E.coli* strain in the culture plate and grown for 24 hours at 37°C and then maintained in 5°C as the mother stock solution. The dish contenting with grown *E.coli* was then washed into the sterilized nutrient broth solution (4.0 g/L) and grows 24 hours at 35°C with gentle air agitation. After growth, the culture was centrifuged for 10 minutes at 7500 G in a benchtop centrifuge (Model SPX; Sorvall). The resulting pellet was washed twice and re-suspended in sterilized 0.05M phosphate buffer solution. Then *E.coli* stock solution was maintained at 5°C.

3.2.3. *Escherichia coli* Enumeration

Selective recovery of *E.coli* was achieved either by specific chemicals in the culture medium or by increasing the incubation temperature above the optimum for growth (Presswood and Strong, 1978). Recovery of stressed coliform bacteria has been of interest because there are lot of evidence showing that stressed organisms do not grow well on the selective media normally used in sanitary microbiology, thereby underestimating the number of surviving microorganisms (Finch *et al.*, 1987). In order to exam the response of bacteria grown under different growth media to freezing and thawing processes, in this project, two kinds of growth media were used.

The *E.coli* were enumerated by membrane filtration using Millipore HAGW047 membrane filter (APHA, 1992). The growth media were the standard m-FC agar (APHA, 1992) and a nonselective agar prepared with the same components as m-FC agar, but excluding 0.01% rosolic acid, which was called Mm-FC (modified M-FC). The performance of media preparation followed the standard method for the examination of water and wastewater (APHA, 1998). In this experiment, the incubation temperature was set at 35°C.

3.2.3.1. Membrane Filtration Procedure

The Standard Method (APHA, 1998) for the preparation of sample dilution and filtration was followed. For each sample, two kinds of media were used.

3.2.3.2. Definitions and Estimation of the Populations of *E.coli*

a). Dead population

The dead population was made up of cells destroyed by freezing, frozen storage and thawing. It is unable to form colonies on a non-selective or selective medium. In other words, the dead population was determinated by the difference between the number of colonies formed on m-FC agar before freezing and after freezing or freeze storage.

b). Injured or damaged population

The injured population is made up of cells damaged by freezing, frozen storage and thawing. The injured cells are unable to form colonies on a selective medium (m-FC agar) but can grow on a non-selective medium (Mm-FC agar). So, the difference between the number of colonies formed on Mm-FC and m-FC media after freezing or freeze storage would present the injured or damaged *E.coli* population.

c). Non-damaged population

The non-damaged population is made up of cells able to form colonies on a selective medium such as m-FC agar after freezing or freeze storage.

d). Viable population

The viable population is made up of colonies that can develop on a non-selective medium such as Mm-FC agar after freezing or freeze storage.

3.2.4. Experimental Design

There are many factors that may affect the survival of microorganisms from freezing and thawing process. Factors that may be involved are showed as following (Lund *et al.*, 2000, and Calcott, 1978):

- type and strain of microorganism;
- nutritional status;
- phase of growth;
- composition of cooling and freezing medium;
- rate of cooling;
- holding temperature;
- time held at low temperature;
- rate of warming to melting point;
- method of determination of viable count; and
- medium used for determination of viable count.

The effect of cooling rate on survival of microorganism had been examed by many researchers (Calcott, 1978; Macleod and Calcott, 1976; Mazur, 1966). The optimum cooling rate for bacteria survival at low cooling rate is between 5 and 40 °C per minutes. Mazur (1966) suggested that at very slow cooling rates, below the optimum rate, extracellular freezing took place and cell damage was mainly caused by exposure to increasing concentrations of solutes. As cooling rates increase, the exposure times to these solute stresses were reduced, with resultant increased cell survival. At higher cooling rates, however, intracellular ice began to form and survival rate fell. Freezing in the presence of NaCl could cause a reduction in survival of *E.coli*, but the response pattern still reflected a cooling-rate-dependent effect (Robinson, 1985).

In general, the longer the period of holding at sub-zero temperature, the lower the survival. The decline in viability is probably caused by continued exposure to the concentrated solution (Robinson, 1985). The rate of thawing was reported have a small effect on survival of bacterial. After rapid cooling, fast warming is more protective than slow warming (Robinson, 1985).

Due to the numerous factors that may influence the survival of bacteria during the freezing and thawing process, a fractional factorial design was selected to determine which factor has significant effect on the *E.coli* survival. In this experiment, 5 factors were selected and two kinds of media were used to determine the viable count. The selected five factors were:

A) freezing temperature (high level: -15°C, low level: -30°C);

B) storage time (high level: 5 days, low level: 0 days);

C) NaCl concentration (high level: 0.85%, low level: null);

D) preserve temperature before freezing (48 to 60 hrs) (high level: 30°C, low level: 5°C); and

E) thawing temperature (high level: 20°C, low level: 5°C).

For the full 2^5 factorial design, there will require 32 runs for testing. A 2^{5-1} fractional factorial design was performed in this experiment, which had only 16 runs for study. Using fractional factorial designs often leads to economy and efficiency in experimentation. Comparing with full factorial design, the fractional factorial design can save half of the works but, at the same time, it cannot get the full information of one main factor, which will be confounded with the high order interaction. From the early studies, the thawing temperature (factor E) had the least effect on *E.coli* survival comparing with other 4 factors. So, thawing temperature was selected to confound with the second order interaction AB (E = AB). If the results suggested that factor E had the significant effect on *E.coli* survival, another half part of experiment would be performed.

The fractional factorial design is shown in Table 3-1.

Table 3-1. 2^{5-1} Fractional Factorial Design

Run	A	B	C	D	E = AB
1	-1	-1	-1	-1	1
2	1	-1	-1	-1	-1
3	-1	1	-1	-1	-1
4	1	1	-1	-1	1
5	-1	-1	1	-1	1
6	1	-1	1	-1	-1
7	-1	1	1	-1	-1
8	1	1	1	-1	1
9	-1	-1	-1	1	1
10	1	-1	-1	1	-1
11	-1	1	-1	1	-1
12	1	1	-1	1	1
13	-1	-1	1	1	1
14	1	-1	1	1	-1
15	-1	1	1	1	-1
16	1	1	1	1	1

After testing following the fractional factorial design, the factors that had significant effects on the *E.coli* survival were selected for further study.

3.2.5. Experiments

3.2.5.1. Batch Test for *E.coli* Reduction

The objective of the batch test was to determine how and which factors could affect the *E.coli* survival after the freeze/thaw process.

In the experiment, the bacteria stock solution was firstly transferred in the sterilized phosphate buffer solution and mixed with a Teflon® – magnetic stir bar for10 minutes. The finial concentration of the *E.coli* solution was 10^6 to 10^7 CFU/mL. After that, a volume of 50 mL mixed solution was placed in 100 mL plastic beakers and covered with sterilized aluminum foil, then put into the freezer. After the solution was frozen, it was taken out and warmed at 5°C for melting. Then enumeration of the density of the surviving microorganisms was performed.

3.2.5.2. *E.coli* Reduction at Different Growth Phase

From early studies, the sensibility of *E.coli* to the freezing was depended on the growth phase of cells. Therefore, in this experiment, the response of *E.coli* in different growth phases to the freeze/thaw process was investigated.

A volume of 1.0 mL of the *E.coli* stock solution was transferred to a 600 mL sterilized growth culture and put into a 35°C water bath. Air was pumped in the flask and the liquid was kept gently stirred. Air was filtrated through a 0.2 µm filter. At the designed time (took sample in every 2 hours in the first 10 hours and then took in every 12 hours), a 50 mL volume of the culture was taken to a sterilized plastic beaker and sealed with aluminum foil and frozen at –35°C for 3 to 4 hours. It was then melted at 5°C and then enumerated for *E.coli* in the liquid.

3.2.5.3. *E.coli* Reduction Versus Freeze/thaw Cycles

The objectives of freeze/thaw cycles test were to identify how did the freezing temperature affect *E.coli* survival and how did the freeze/thaw cycles affect the *E.coli* reduction.

The bacteria stock solution was firstly transferred into the sterilized phosphate buffer solution in room temperature and mixed with a Teflon® – magnetic stir bar for10 minutes. The finial concentration of the *E.coli* solution was 10^6 to 10^7 CFU/mL. After that, a volume of 50 mL mixed solution was placed in 100 mL plastic beakers and covered with sterilized aluminum foil, then put into the freezer. After the solution was frozen, it was taken out and warmed at 5°C for melting. After 12 to 14 hours, all of the ice had been melted and then enumerated the density of the surviving microorganism. After that, the samples would be recovered with aluminum foil and put back into the freezer for the next freeze/thaw cycle. For each *E.coli* solution, 5 to 6 freeze/thaw cycles were performed.

3.2.5.4. Spray Freezing Test for *E.coli* Reduction

In this spraying test, water sample was atomized through a nozzle and sprayed into atmosphere. Because the water drops became very small, the freezing rate would become higher than the batch test. Part of the bacteria and impurities would be rejected to the unfrozen water. The objective of the spraying test was to study whether the freezing rate and freezing behavior had significant effects on *E.coli* inactivation and to compare the results with those of batch tests.

The spraying test was carried out by using a diaphragm pump (Model: 8000-813-238, Garden Grove, CA) spraying the water sample in a container, which was placed in a −15°C freezer. The orifice size of nozzle (Evenmist™, Field Controls, NC) was 0.65 mm and opened in 3 directions with 60° angle. In this experiment, the pressure of the pump was set at 100 psi and the flow rate was set at 20 and 24 mL/minute. All of the spraying freezing tests were performed in a cooling room with the room temperature from 1 to 5°C.

Samples were prepared by transferring the bacteria stock solution in the sterilized distilled water (5°C) and completely mixing for 10 minutes. The finial concentration of the *E.coli* solution was 10^6 to 10^7 CFU/mL. The pipeline was rinsed by the 100 mL/L bleach solution for 10 minutes and then rinsed with sterile water for 15 minutes and held for half hour. Before the spraying test, the pipeline was washed with the sample solution for 10 minutes. Then, the water samples were sprayed into the freezer (-15°C) for 45 minutes at flow rate of 24 mL/minute or 15 minutes at 20 mL/minute. The experimental set up for spraying test was illustrated in Figure 3-1.

The ice and unfrozen solution were collected in a tray. After the spraying, the tray was taken out and the ice and unfrozen solution were collected, half of the ice was stored at -15°C for 2 days and other half of the ice was heated and melted under a lamp (150 W) located about 200 mm above the ice surface under the cooling room temperature. For each sample, the melted ice water was collected for 3 to 4 times throughout of the melting process and each was got at about 10% to 30% of the total ice volume was melted. After the collection, the melt water was tested for *E.coli* enumeration. Then, the concentration of microorganisms in the melting ice and unfrozen water sample were determined. After the test, the pipeline was washed with 100 mL/L bleach solution for 20 minutes. This was followed by rinsing with sterile water for 15 minute.

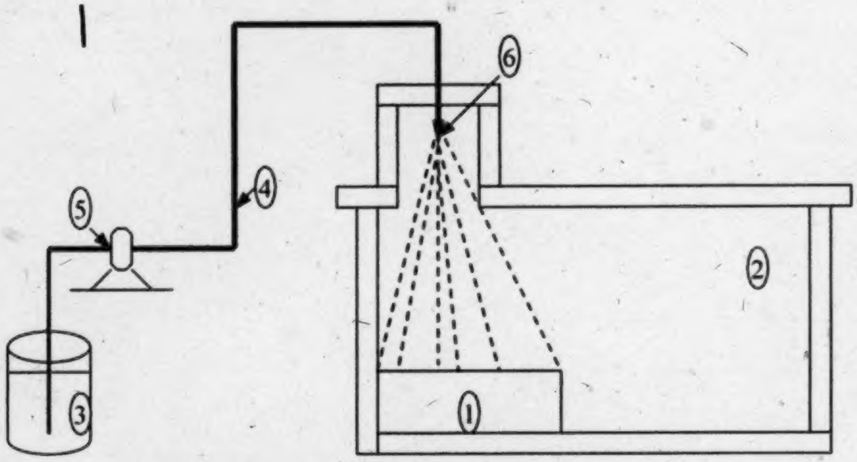


Figure 3-1. Schematic of Spraying Test Apparatus

1. Ice collection tray. 2. Freezer (-15°C)
3. Water sample. 4. Pipeline
5. Pump 6. Nozzle

3.2.5.5. Scanning Electron Microscope Test

Scanning Electron Microscope (SEM) was used in this experiment to detect the cells structure change after freezing/thawing process.

The images of the microorganisms in liquid phase were conducted by Dr. Ming Chen in Surgical-medical Research Institute of University of Alberta. The SEM test was carried out by a Scanning Electron Microscope machine (Hitachi S-2500, Japan).

1.0 mL stock *E.coli* solution was mixed in 100 mL sterile buffer solution to make the *E.coli* concentration of 10^6 to 10^7 CFU/mL. Then the sample was frozen at −15°C for 7 to 8 hours and melted at 5°C. After that, the melted *E.coli* sample was left on a poly-L-Lysine coverslip (Sigma, USA) for half hour and the bacteria would be attached on the slip. The samples were then fixed in 2.5% glutaraldehyde in Milloning's buffer (PH = 7.2) for 1.0 hour and post-fixed with 1% OsO_4 (osmium tetroxide) at the same buffer at room temperature for 1.0 hour. Then the samples were dehydrated in a serial graded ethanol (50%, 70%, 90% and 100%) for 10 minutes each. After that, the samples were critical point dried at 31°C for 5 minutes. Then the coverslips were mounted on stubs for gold coating with a sputter coater (Edward, model S150B sputter coater, Japan). Finally, the samples were put into the specimen chamber of scanning electron microscope and examined using an acceleration voltage of 1.5 or 2.0 KV.

The images of the microorganisms in ice phase were taken by Mr. George Braybrook at Earth and Atmospheric Science department of University of Alberta using a Scanning Electron Microscope machine (JOEL JSM-6301F, Japan) with a cryosystem attachment (Emitek, K1250, UK).

1.0 mL stock *E.coli* solution was mixed in 100 mL sterile water solution to make the *E.coli* concentration of 10^6 to 10^7 CFU/ml. A plastic straw was placed in the water sample and then frozen at −15°C for 7 to 8 hours. When the ice water sample with the plastic straw was taken out of the freezer, put into an insulated container and immediately taken to the SEM lab.

In the SEM lab, the straw containing the ice water sample was taken out of the container and submerged in liquid nitrogen for about 10 minutes to produce a clean undisturbed surface. After cooling, the plastic straw was cut to a 2 to 3 cm length, fixed to the sample holder with Tissue-TEK O.C.T. compound (#27050, Ted Pella Inc. USA) and transferred into the cryo chamber where the sample was gold coated. The sample was sputter coated with gold for a total thickness of 100 Angstroms. Following coating, sample was put into the specimen chamber of scanning electron microscope and examined using an acceleration voltage of 5.0 KV.

3.2.6. Statistical Analysis

Two kinds of statistical analysis softwares were used in the data analysis. One was SPSS® 12.0 for analyzing 3 factors with multi-levels designs. Other analysis were done by using Microsoft® Excel.

3.3. Results and Discussion

3.3.1. Fractional Factorial Experiment Design

The data collected during the experiment are reported in Appendix A. Using the function of "Regression" in Microsoft Excel, the factors that had significant effects on the *E.coli* removal were determined. The ANOVA and regression results are shown in Tables 3-2 and 3-3.

p-vale, called significant level, was defined as the smallest level of significance that would lead to rejection of the null hypothesis (Montgomery, 2001). In this fractional factional design, the null hypothesis was that the effect of each factor was significant. From the results, the storage time (B) (p-value = 0) and NaCl presence (C) (p-value = 0.033 for m-FC) had the most significant effects on the survival of *E.coli*. All of the second order interactions had insignificant effects to the *E.coli* reduction. Two methods, m-FC and Mm-FC, yielded the same conclusion. Even with the Modified m-FC method, the p-value of factor D was equaled to 0.074 that was not significant at 95% confidence level, but it was very close to the significant level ($\alpha = 0.05$), the preserve temperature (D) was regarded as having important effect to the viability of *E.coli*. The ANOVA and regression results for second order interaction are presented in Appendix B.

Table 3-2. Summary of Regression Statistics for m-FC Method

ANOVA	*df*	*SS*	*MS*	*F*	*Significance F*	
Regression	5	4.593	0.919	8.058	0.003	
Residual	10	1.140	0.114			
Total	15	5.734				
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.932	0.084	11.045	0.000	0.744	1.120
Freezing temperature	0.027	0.084	0.322	0.754	-0.161	0.215
Storage time	0.455	0.084	5.388	0.000	0.267	0.643
NaCl Concentration	0.208	0.084	2.469	0.033	0.020	0.396
Preserve temperature	-0.189	0.084	-2.245	0.049	-0.378	-0.001
Melting temperature	0.012	0.084	0.139	0.892	-0.176	0.200

Table 3-3. Summary of Regression Statistics for Modified m-FC Method

ANOVA	*df*	*SS*	*MS*	*F*	Significance F	
Regression	5	3.728	0.746	7.949	0.003	
Residual	10	0.938	0.094			
Total	15	4.665				
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.850	0.077	11.105	0.000	0.680	1.021
Freezing temperature	0.003	0.077	0.042	0.967	-0.167	0.174
Storage time	0.407	0.077	5.321	0.000	0.237	0.578
NaCl Concentration	0.208	0.077	2.721	0.022	0.038	0.379
Preserve temperature	-0.153	0.077	-1.998	0.074	-0.324	0.018
Melting temperature	0.016	0.077	0.206	0.841	-0.155	0.186

The early studies had indicated that the death of bacteria was related to the time held at a frozen temperature (Lund *et al.*, 2000; Robinson, 1985; Calcott, 1978). Storage death maybe caused by crystal growth due to re-crystallisation (Davies, 1970), by different precipitation of solutes with time (Van den Berg, 1968) and by continued exposure to the concentrated solution (Robinson, 1985). In this experiment, the storage time (B) was the most important factor in the effecting *E.coli* survival. The coefficient of B was positive, so the longer storage times the higher *E.coli* reduction.

The experimental results also indicated that salt concentration had a significant effect on the survival of *E.coli*. The coefficient of C was 0.208, which means that the higher NaCl concentration had more lethal effects to *E.coli* survival. Because freezing in the saline solution would make the injured *E.coli* exposure to the concentrated NaCl solution in the unfrozen portion. The difference of NaCl concentration between the cells and the unfrozen liquid would result in the diffusion of water from the microbial cells that would make the cells dehydrate and shrink.

Factor D, the preserve temperature, also had a significant impact on the *E.coli* survival. From the regression analysis, the coefficient of D was negative that means the low environmental temperature will make *E.coli* more sensitive to the freeze/thaw process. When the bacteria grew or were kept at a lower temperature, they would become more sensitive to the freezing. Low temperature could retard chemical reaction, enzyme action, and the growth of microorganisms. It could also affect the membrane structure and function and cause a change in membrane fatty acid composition (Lund, 2000). The net effect of these changes could affect the cell structure and further influence its ability to resist the effect of freezing. For *E.coli*, the optimum growth temperature is 39°C and the minimum temperature for growth is 4°C (Frazier and Westhoff, 1988). The cells kept at warmer temperatures are more viable and stronger than those at the lower temperatures. Therefore, *E.coli* preserved in higher temperature environments become more resistant to the freeze/thaw process.

From the results of fractional factorial design, a linear model can be established: for m-FC method: $Y = 0.932 + 0.455 \cdot X_B + 0.208 \cdot X_C - 0.189 \cdot X_D$ and for modified m-FC method: $Y = 0.85 + 0.407 \cdot X_B + 0.208 \cdot X_C - 0.153 \cdot X_D$. Checking the residual, the variances of three factors were constant versus the residuals and also the residuals fit the normal distribution. So those estimate modals are suitable to predict the result in the select range of this experiment. The data analysis can refer to Appendix B.

For better understanding the effects of freezing, the influence of cell growth phases, storage times and freezing cycles on the *E.coli* survival were examined.

3.3.2. Effect of Freezing on the Survival of *E.coli* at Different Growth Phase

This experiment was carried out by incubating *E.coli* in a 2.0 mg/L nutrient broth culture and then the culture was frozen at -35°C. In different growth phases, the response of *E.coli* to freezing varied greatly. The *E.coli* growth and reduction at exponential and stationary phase (with Mm-FC method) are presented in Figure 3-2 and Figure 3-3. The detailed information about the *E.coli* reduction in different growth phases is provided in Appendix A.

As illustrated in Figure 3-2, in the exponential growth phase (From A to B), cells are much more sensitive to freezing-thawing stress than those in the stationary growth phase (After point B). The highest inactivation rate in the log phase reached 4 log units. From Figure 3-3, in the stationary growth phase (From point B to C), the inactivation rate was just 0.5 to 1.0 log units. When the cells were in the decay phase (After point C), the *E.coli* become sensitive to the freezing again and the freezing/thawing process can inactivate 4 log units. This phenomenon had been reported by earlier studies (Robinson, 1985; Calcott, 1978; Ray and Speck, 1973) but the mechanisms of growth phase related changes in cryosensitivity are still not clear. It may be explained that during the stationary phase, the cells grow mature and the structure becomes stronger than in other phases, therefore, the cells are most resistant to the freezing.

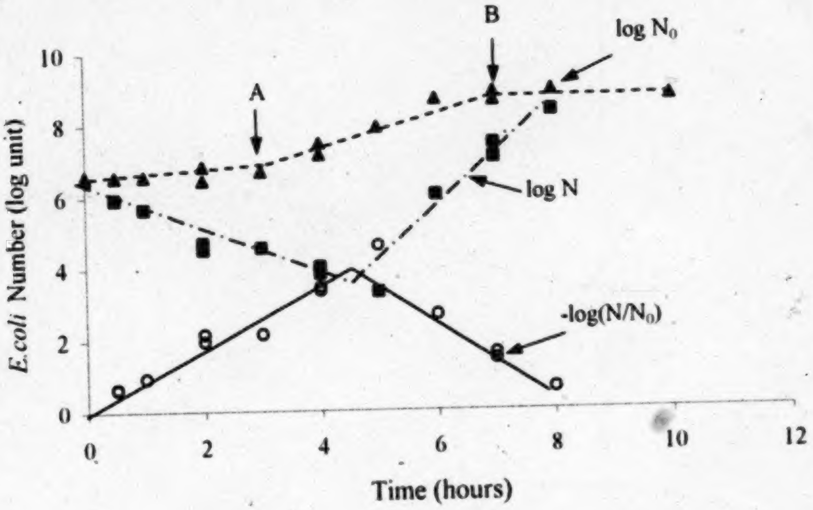


Figure 3-2. *E.coli* Reduction in Exponential Phase.

log N_0--Initial *E.coli* number; log N-- *E.coli* number after freezing; log N/N_0-- *E.coli* reduction

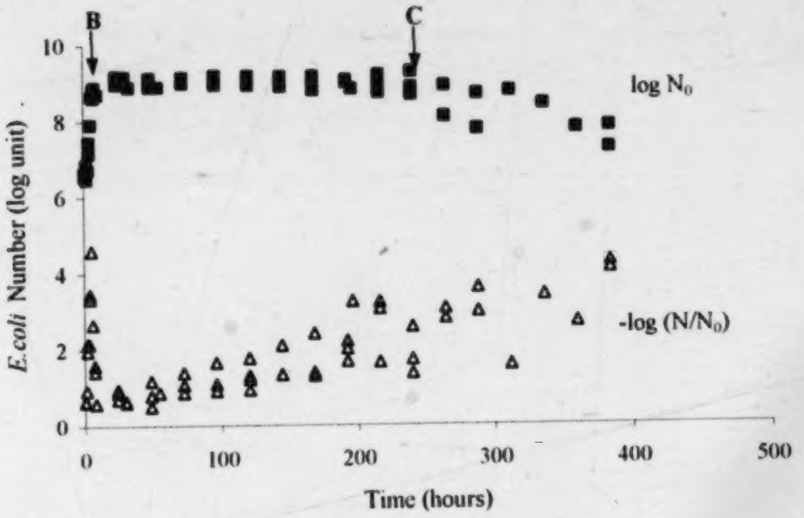


Figure 3-3. *E.coli* Reduction in Growth Phase.
log N_0--Initial *E.coli* number; log N/N_0-- *E.coli* reduction

In this experiment, two test media were used for *E.coli* count determination, m-FC agar and m-FC agar eliminating rosolic acid. Using "General linear model" in SPSS to analyze the *E.coli* number in stationary growth phase and to compare the two test methods. The p-value of the test method in comparing the *E.coli* reduction was 0.996 that indicated that there were non-significant differences between two media for the *E.coli* counts for the samples without freezing. And at the same time, after freezing, there were significant differences in the *E.coli* reduction between the two methods. The average difference between the two methods for *E.coli* reduction was 0.18 log units. The results and data analysis are reported in Appendix A and B. Because the role of rosolic acid is to suppress the growth of stressed cells, after freezing, the difference of two methods should represent the stressed but not dead cells number.

3.3.3. Effect of Freezing Storage Time on *E.coli* Survival

Various experimental results indicated that the storage time and temperature could affect the viability of *E.coli* after freezing. After freezing, death is initially fairly rapid, particularly at –2°C to -5°C, and then gradually slows on holding at a constant sub-zero temperature until eventually, in the late stages of storage, viable numbers remain almost constant (Ingram and Mackey, 1976).

For this project, the effect of freezing storage time on *E.coli* is listed in Table A-5 to A-10 of Appendix A. The *E.coli* inactivation with freezing storage time at different temperature is presented in Figure 3-4 and 3-5.

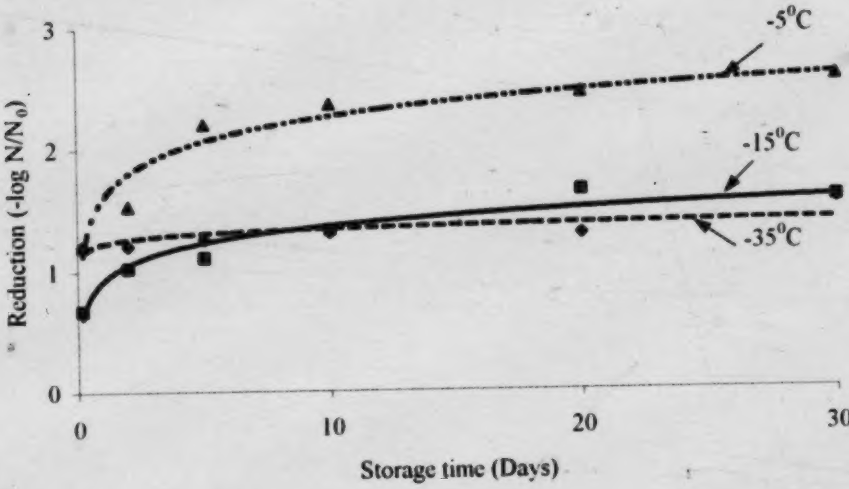


Figure 3-4. *E.coli* Reduction vs. Storage Time (m-FC Method)

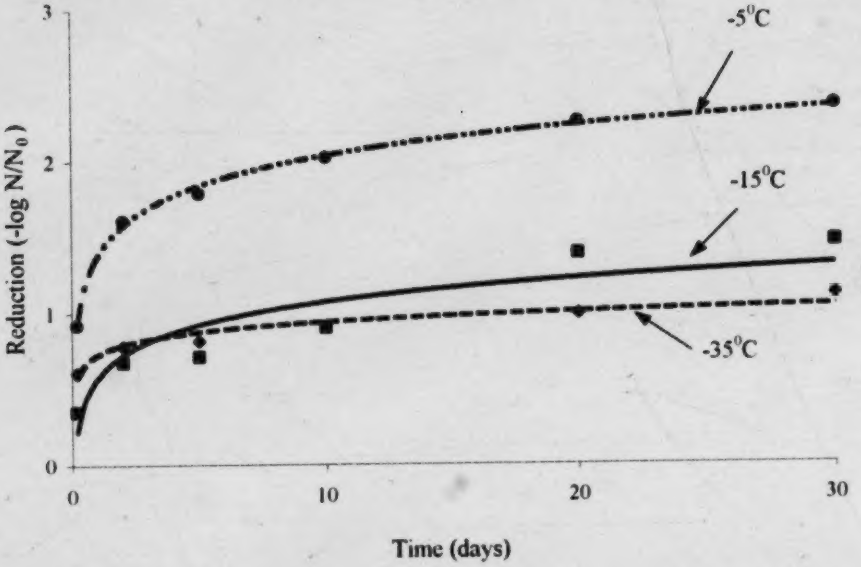


Figure 3-5. ***E.coli*** **Reduction vs. Storage Time (Modified m-FC Method)**

The experimental results revealed that with the same storage time, the warmer freezing temperature had more lethal effects on *E.coli* survival. At a freezing and storage temperature of -5°C, the *E.coli* inactivation was from 2.1 to 2.6 log-units in 30 days storage for both test methods, which was higher than that at –15 °C and -35°C. For the temperatures –15 and -35°C, at the beginning, cells frozen at -15°C had higher survival rate than those at -35°C and about 4-5 days later, the cell death become more rapid than that at -35°C with increasing storage time. It suggested that the organisms lose viability when held at or just below freezing temperature. When the holding temperature was lowered, the death rate reduced and below -60°C the rate of death would be very low or zero (Macleod and Calcott, 1976). Sanin *et al.* (1994) found that with the same storage time, the freezing temperature could affect the reduction in fecal coliform numbers and somewhat enhance the inactivation of *Salmonella spp.*, the warmer the temperature, the higher the reduction rate. Virus removal, which was measured by plaque forming units, was not affected by the freezing temperature.

Using the General Liner Model in "SPSS" program for the data analysis, the conclusion was that three factors, test methods, freezing temperature and storage time, all had significant effects on the *E.coli* reduction. The results are reported in Table 3-4. The detailed output information is provided in Appendix B.

Table 3-4. Univariate Analysis of Variance for Freezing Storage Test

Tests of Between-Subjects Effects -- Dependent Variable: Removal ($-\log N/N_0$)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	53.494(a)	35	1.528	15.720	0.000
Intercept	316.639	1	316.639	3256.74	0.000
Methods	4.428	1	4.428	45.548	0.000
Temperature	25.048	2	12.524	128.815	0.000
Time	17.737	5	3.547	36.486	0.000
Methods * temperature	0.296	2	0.148	1.525	0.221
Methods * Time	0.265	5	0.053	.546	0.741
Temperature * Time	4.591	10	0.459	4.722	0.000
Error	13.806	142	0.097		
Total	362.746	178			
Corrected Total	67.300	177			

(a) R Squared = 0.795 (Adjusted R Squared = 0.744)

The two test methods, m-FC and Mm-FC, have the same trend for the *E.coli* removal with the increasing of storage time, but the removal rates were different. Because the m-FC method can suppress the growth of stressed cells on the selective media, the non-lethally freeze-injured cells could fail to develop colonies in the presence of selective agents and thus the removal rate was higher than that of the modified m-FC method. The difference between two methods should represent the injured cells number. Table 3-5 and Figure 3-6 present the percentage of injured cells at different freezing temperatures.

Table 3-5. Average Injured *E.coli* Percent at Different Temperatures

Storage time	Cells Injured Percent (%)		
(days)	Storage at -35°C	Storage at-15°C	Storage at -5°C
0	18.16	22.36	4.30
2	10.40	11.96	0.00
5	9.88	13.98	0.71
10	7.26	9.29	0.22
20	5.07	1.32	0.00
30	5.50	0.10	0.03

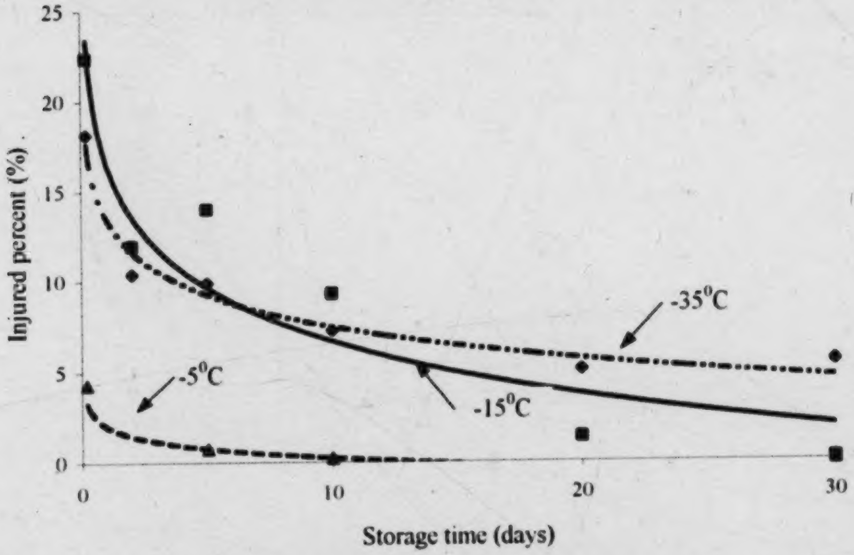


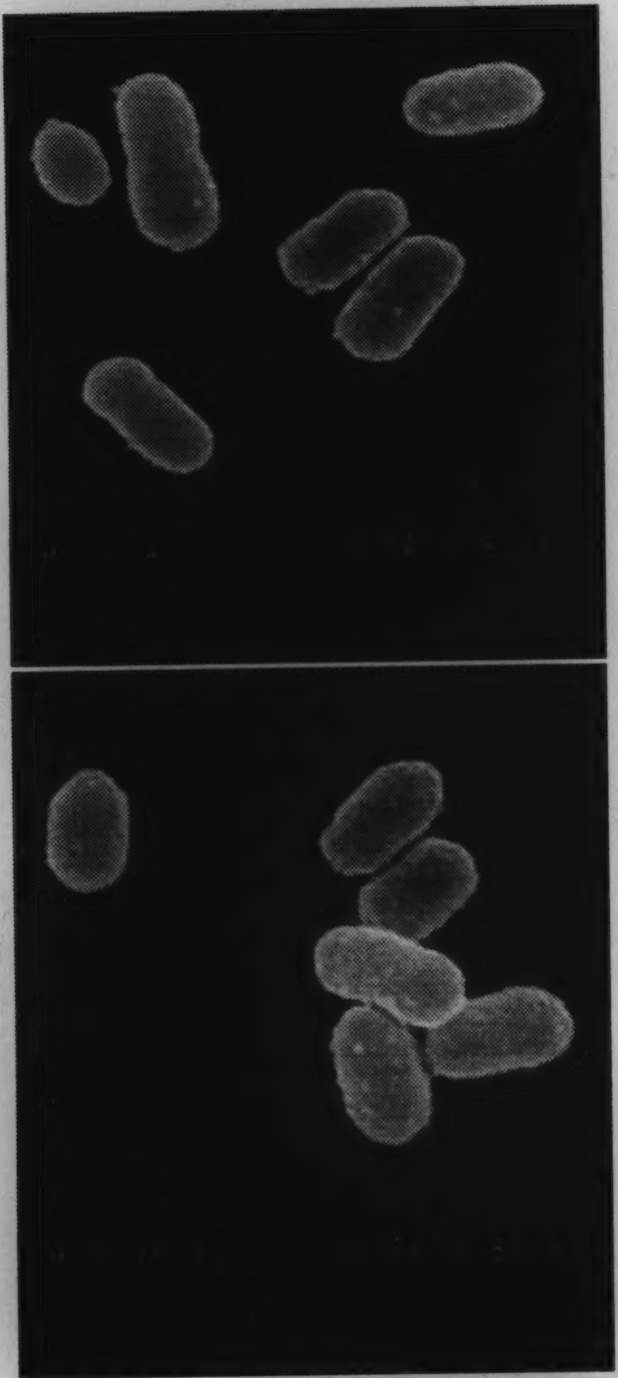
Figure 3-6. Injured *E.coli* Percent During Storage

Some cells that had suffered of non-lethal damage during freezing could recover if they were in a suitable environment. At low cooling rates of freezing, ice forms extracellularly and make cells dehydrate and shrink. So the lethal effects result from damage of the protective cell barriers (Calcott, 1978), leakage of cellular materials, and the high concentrations inside the cells (Robinson, 1985).

With increasing storage time, the number of injured cells would decrease. That is probably due to the continued exposure to the cold environment of injured cells and cellular materials leakage slowed. In the late stages of storage, the viability of the number of *E.coli* remained almost constant.

At –5 °C, the percent of the non-lethally freeze-injured *E.coli* were much lower than at –15 °C and -35 °C. It was probably that when frozen at warmer temperatures, the cells had longer time of exposure to the concentrated solutes. Then the injured cells continued to lose their cellular materials and eventually lead to death. At -35 °C, at the beginning of the freezing, the *E.coli* survival was lower than -15 °C. Although the cooler temperature could cause more shock impact to the cells, but these impacts might not result in lethal damage to cells due to the short exposure time to the concentrated solution. In earlier studies, they had found that below –60 °C the rate of death was usually very low or zero (Macleod and Calcott, 1976). In this experiment, after long time storage, the injured cells' number was higher at -35°C than that at -15 °C.

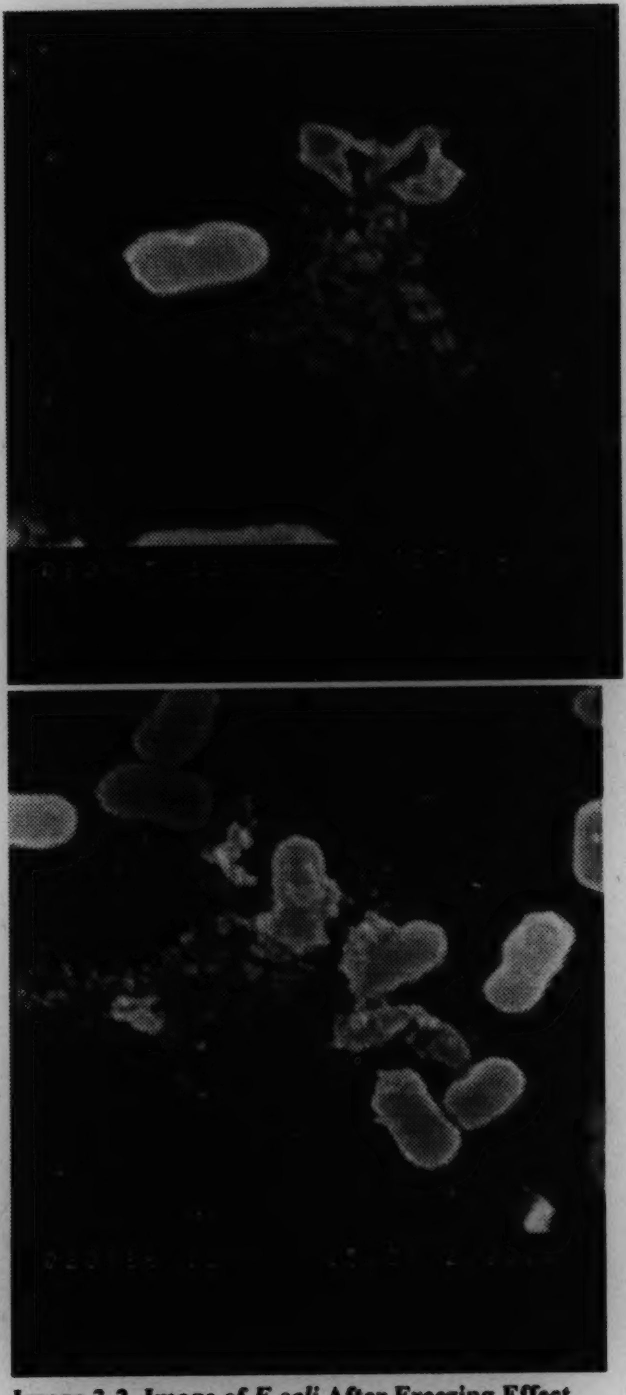
The following SEM images (from Image 3-1 to 3-4) show the change of the *E.coli* structure before and after freezing. Image 3-1 shows the healthy *E.coli* cells before freezing. Image 3-2 and 3-3 present the damaged *E.coli* cells after freezing. Image 3-4 is the *E.coli* in the ice structure. From the SEM images, Images 3-2 to 3-3, some of the *E.coli*'s cell walls had been damaged by the freezing and some were broken and cellular materials leaked out.



(20,000 ×)

(20,000 ×)

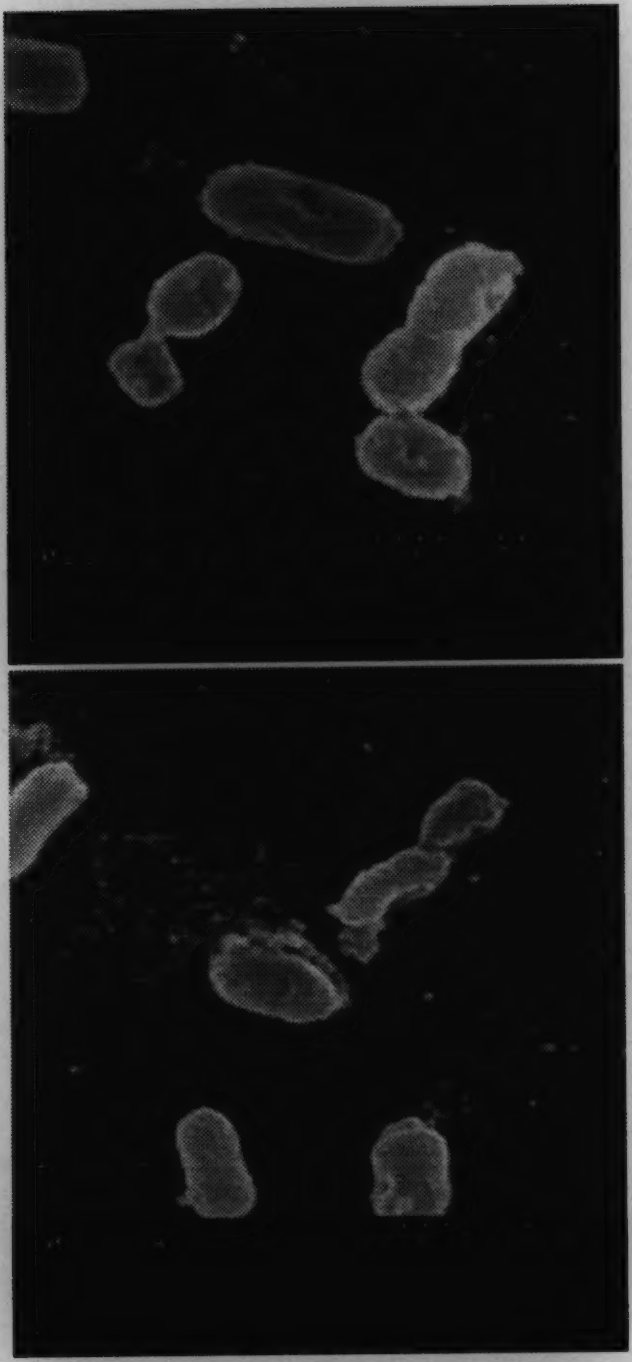
Image 3-1. Image of *E.coli* Without Freezing Effect



(20,000 ×)

(20,000 ×)

Image 3-2. Image of *E.coli* After Freezing Effect



(20,000 ×)

(20,000 ×)

Image 3-3. Image of *E.coli* After Freezing Effect

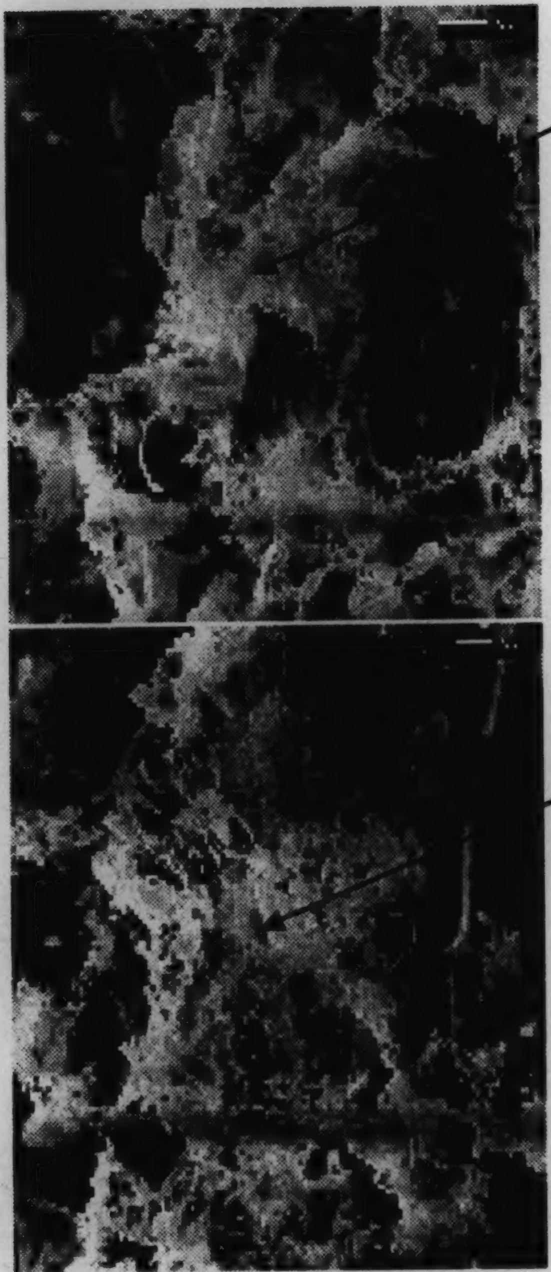

E.coli
(8000 ×)
E.coli
(8000 ×)

Image 3-4. Image of *E.coli* in the Ice

3.3.4. The Effect of Freezing Cycles on *E.coli* Survival

The freeze/thaw process can damage the cell structure and then inactivate the bacteria. It can be expected that with the increased number of freezing cycles, the number of inactivated *E.coli* would continue decline. The *E.coli* removals at different temperatures up to five freeze/thaw cycles are shown in Table 3-6 and Figure 3-7 to 3-8.

Table 3-6. *E.coli* Reduction for the Freezing Cycles at Different Temperatures

	Modified m-FC method				m-FC method			
Freezing cycles	*E.coli* Reduction at -35°C				*E.coli* Reduction at -35°C			
	(%)	Stdev*.	$-\log (N/N_0)$	Stdev*.	(%)	Stdev*.	$-\log (N/N_0)$	Stdev*.
1	73.98	5.74	0.59	0.10	91.20	5.60	1.13	0.33
2	93.79	3.70	1.25	0.24	96.91	1.62	1.55	0.23
3	98.84	0.28	1.94	0.10	98.93	0.74	2.03	0.33
4	99.26	0.48	2.18	0.30	99.56	0.36	2.44	0.41
5	99.85	0.15	2.98	0.56	99.90	0.09	3.13	0.47
Freezing cycles	*E.coli* Reduction at -15°C				*E.coli* Reduction at -15°C			
	(%)	Stdev*.	$-\log (N/N_0)$	Stdev*.	(%)	Stdev*.	$-\log (N/N_0)$	Stdev*.
1	52.92	9.10	0.38	0.10	78.15	5.28	0.69	0.10
2	87.47	2.00	0.88	0.07	92.81	1.44	1.12	0.09
3	95.82	5.36	1.26	0.39	97.17	3.13	1.48	0.44
4	96.63	2.82	1.38	0.30	99.05	3.12	1.84	0.66
5	99.65	2.82	2.14	0.82	99.81	0.09	2.75	0.22
Freezing cycles	*E.coli* Reduction at -5°C				*E.coli* Reduction at -5°C			
	(%)	Stdev*.	$-\log (N/N_0)$	Stdev*.	(%)	Stdev*.	$-\log (N/N_0)$	Stdev*.
1	86.89	1.20	0.88	0.04	88.87	1.52	0.96	0.06
2	99.08	0.66	2.14	0.39	98.96	0.59	2.05	0.33
3	99.20	0.69	2.21	0.38	99.41	0.44	2.33	0.36
4	99.76	0.14	2.70	0.30	99.79	0.13	2.74	0.29
5	99.94	0.05	3.50	0.75	99.94	0.04	3.28	0.26

* Standard Deviation

In this experiment, the inactivation rates increased with the increase of freeze/thaw cycles and the death rate was the highest at -5 °C. But the removal rates at -

15 °C were lower than at -35 °C. One reason for the death of *E.coli* is at low cooling rates, ice forms extracellularly and cells dehydrate and shrink. Under these conditions, lethal effects result from high solute concentrations inside the cells (Mazur, 1966). Another one was probably due to the cold shock, which also can damage the cell structure. At -5 °C, the exposure time to the concentrated solution was the longest but the cold shock from the temperature drop was the minimal. For -35 °C, the situation was reversed. Therefore, the exposure time or cooling rate was more critical to the survival of *E.coli* than the cold shock. When the two factors' effects were minimal, the cooling rate was optimum for bacteria survival. In this experiment, at -15 °C, the viability of *E.coli* was the highest in every freezing cycle.

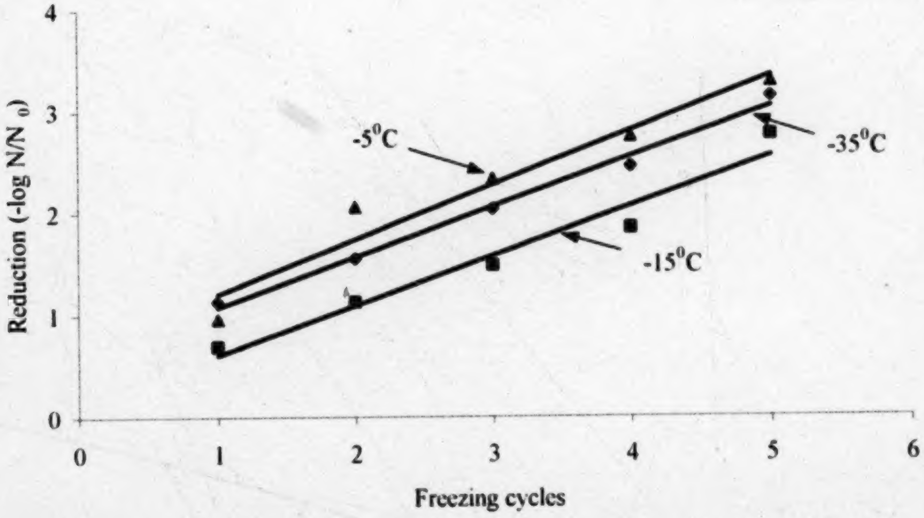


Figure 3-7. ***E.coli*** **Reduction vs. Freezing Cycle (m-FC method)**

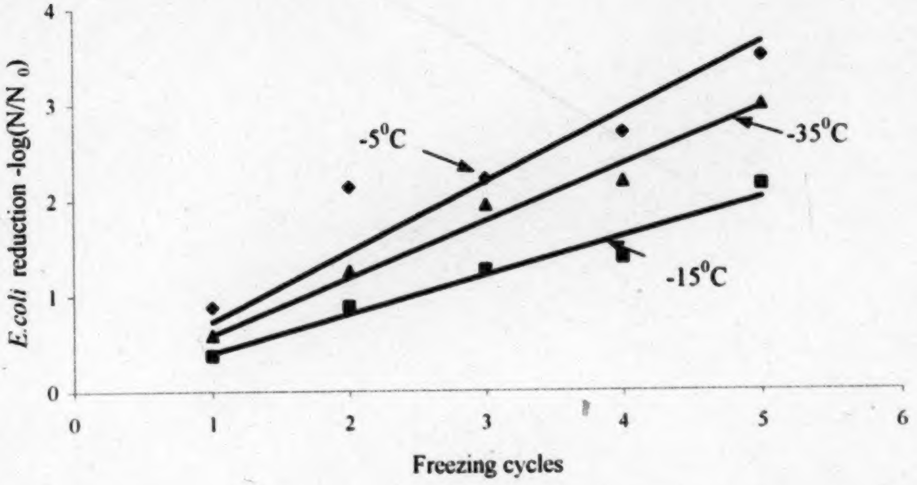


Figure 3-8. *E.coli* Reduction vs. Freezing Cycle (Modified m-FC method)

Using the General Linear Model in "SPSS" program doing the data analysis. The results are reported in Table 3-7 and the detailed results can refer to Appendix B.

Table 3-7. Univariate Analysis of Variance for Freezing Cycle Test

Tests of Between-Subjects Effects (Dependent Variable: Removal)					
Source	Sum of Squares	*df*	Mean Square	F	Sig.
Corrected Model	117.884(a)	34	3.467	22.613	**0.000**
Intercept	428.649	1	428.649	2795.686	**0.000**
Methods	1.201	1	1.201	7.832	**0.007**
Temperature	19.513	2	9.757	63.634	**0.000**
Cycle	72.936	5	14.587	95.139	**0.000**
Methods * Temperature	0.763	2	0.381	2.488	**0.090**
Methods * Cycle	0.127	5	0.025	0.165	0.974
Temperature * Cycle	3.624	10	0.362	2.364	**0.018**
Methods * Temperature * Cycle	0.360	9	0.040	0.261	0.983
Error	11.039	72	0.153		
Total	564.896	107			
Corrected Total	128.923	106			

(a) R Squared = 0.914 (Adjusted R Squared = 0.874)

As indicated in Table 3-7, three factors, test methods, freezing temperature and freeze/thaw cycles, have significant effects on the *E.coli* reduction. The interaction effect of temperature and cycle was also significant. That means the reduction rate of *E.coli* did not grow with the temperature increase. In this experiment, at –15 °C, the reduction rate was the lowest. At the same time, the interaction effect of temperature and methods could also affect the reduction rate. So, at different freezing temperatures, the cells injured and death rate should be different. Figure 3-9 shows the percent of injured cells in the freezing cycles test.

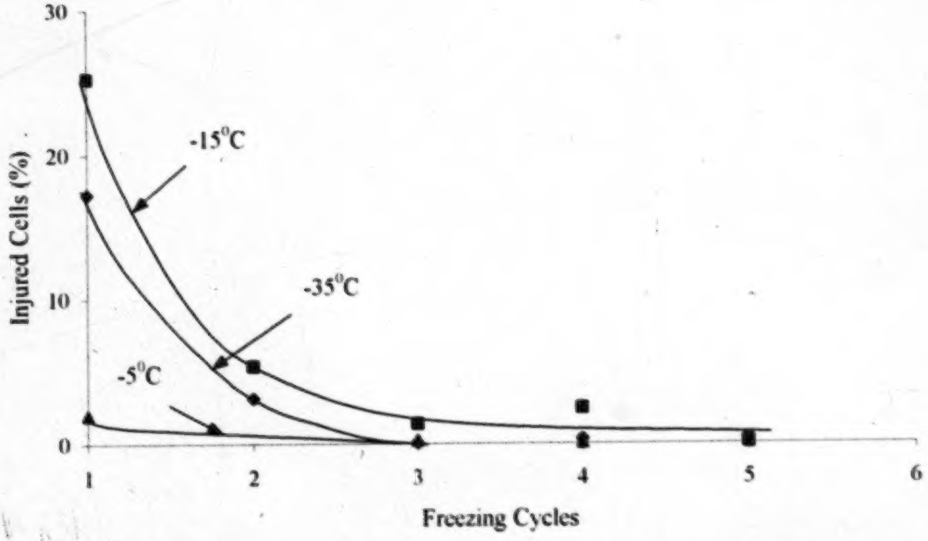


Figure 3-9. Percentage of Injured *E.coli* Cells vs. Freeze/Thaw Cycles

At -5 °C, the percent of non-lethal injured cells numbers were very low, which indicated most of *E.coli* reduction was lethal. At cold temperatures, the cells damage was caused by two effects—cold shock and exposure time to concentrated solution. At -15 °C, both of the two effects were not high and caused more non-lethal damage to cells than the two other freezing temperatures. Because the injured cells were easier to be killed than others, the injured cells number decreased and tended to zero with the cycle time increased.

3.3.5. Spraying Freezing Test for *E.coli* Reduction

The results of spraying freezing tests are reported in Appendix A. The two spraying times had no significant effect on *E.coli* reduction in the melted ice water. The data analysis is reported in Appendix B. The average reduction rate was 0.30 to 0.35 log-units for the melted ice water. From the experimental results, the two test methods had no significant difference for *E.coli* reduction. The results of paired t-test are presented in Table 3-8.

Table 3-8. Paired t-test for *E.coli* Reduction of Two Test Methods

	m-FC	Modified m-FC
	Reduction (-log N/N_0)	
Mean	0.328	0.314
Variance	0.009	0.004
Observations	7	7
Pearson Correlation	0.556	
Hypothesized Mean Difference	0	
df	6	
t Stat.	0.470	
P(T<=t) one-tail	0.327	
t Critical one-tail	1.943	
P(T<=t) two-tail	**0.655**	
t Critical two-tail	2.447	

From the results, the p-value was 0.655 that suggested the two test methods had no significant difference for *E.coli* reduction. This means, during spraying freezing test, that most of damages were lethal to the *E.coli*.

Because the freezing time was shorter (less than 1 hour) than the batch test, the death rate of spraying test was lower than batch test. The results of t-test are presented in Table 3-9.

Table 3-9. t-test for *E.coli* Reduction of Spraying and Batch Tests

	Spaying Test	Batch Test (Mm-FC)	Batch Test (m-FC)
	Reduction (-log N/N_0)		
Mean	**0.314**	**0.361**	**0.708**
Variance	0.004	0.008	0.014
Observations	7	10	9
Hypothesized Mean Difference		0	0
df		15	13
t Stat		-1.257	-8.525
P(T<=t) one-tail		0.114	0.000
t Critical one-tail		1.753	1.771
P(T<=t) two-tail		0.228	0.000
t Critical two-tail		2.131	2.160

From the data analysis, there were significant differences between the spraying test and the batch test with m-FC methods. A possible explanation was that in the spraying test, the shorter time for *E.coli* to expose to the surrounding solution made the *E.coli* reduction rate decrease.

In the runoff water sample (unfrozen water after spraying), the *E.coli* concentration had got higher than the original water sample. In this experiment, when the spraying time is short (about 15 minutes), the average of *E.coli* concentration in the runoff water could increase about 23% for m-FC method and 52% for the Mm-FC method. There have been numerous studies showing that impurities are rejected by ice crystal formation (Gao, 1998). When ice is crystallized from an aqueous solution, the ice crystal is essentially built up by pure water, leaving the solutes in the remaining liquid phase (Gay, et al., 2003). So, after spraying, the solutes and *E.coli* would be rejected into the unfrozen water and then made the concentration of *E.coli* in the runoff got increase. But when the spraying freezing test kept long time (40 to 45 minutes), the *E.coli* concentration had no significant different between the runoff and the original water sample. That may be caused by the long time exposing to the low temperature and concentrated solution. Because, after spraying freezing test, the cells had been injured or damaged and the long time exposing to the low temperature and concentrated solution had become lethally to *E.coli* survival.

In this experiment, the rejection of *E.coli* from the ice by the ice crystal process was also proven by the accumulative melting test after spray freezing. At the beginning of ice melting, cells were rejected from the ice structure and *E.coli* got concentrated. There was no *E.coli* reduction in the first 10% melt water. In the late phase, the *E.coli* reduction could reach to 0.7 log units and the average reduction rate in spray freezing test was just 0.3 to 0.4 log units. The data of results were list in Appendix A and the trend of *E.coli* rejection versus the accumulative melting water percent can refer to Figure 3-10.

After spraying test, part of ice samples had been stored in –15 °C for 2 days and found that the removal rate for these samples increased greatly. The results were reported in Appendix A. Figure 3-11 compares the differences of *E.coli* reduction between the two ice treatments.

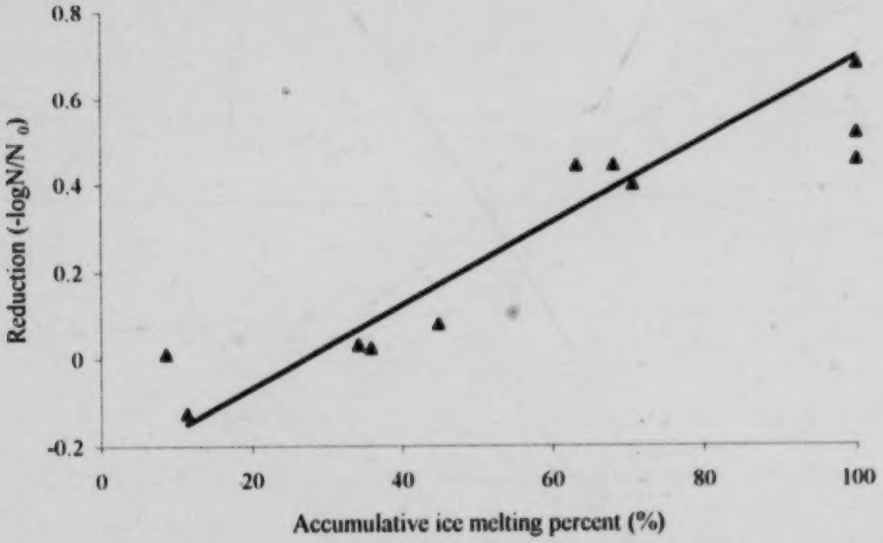


Figure 3-10. ***E.coli*** **Reduction vs. Accumulative Melt Ice Percent**

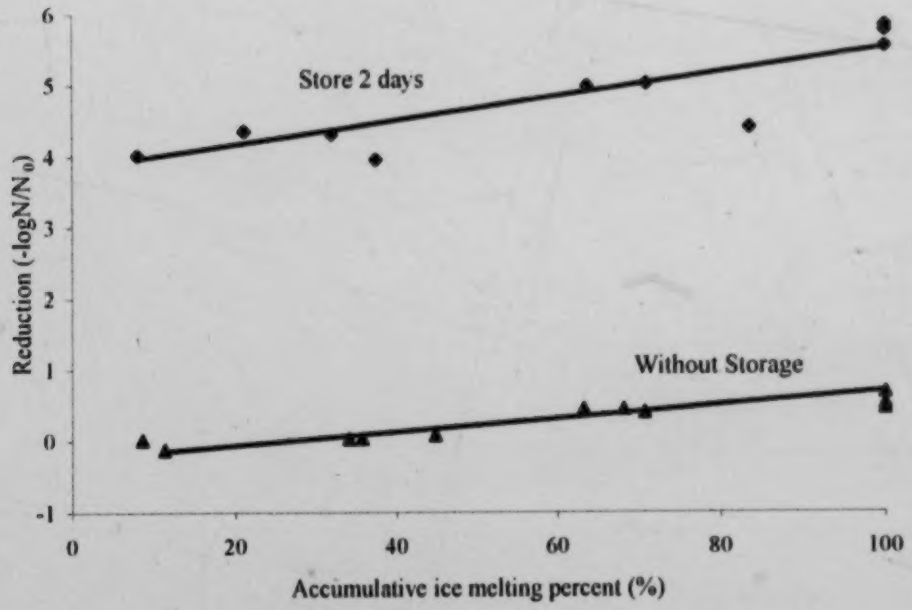


Figure 3-11. Comparison of ***E.coli*** **Reduction for Ice Storage**

After storing for 2 days, the death rate became much higher than those of without storage and the average inactivation rate for spraying freezing test could reach to 4.56 log units. In the spraying test, the cooling rate was much higher than the batch tests. Under the high cooling rates, the lethal effects mainly came from intracellular ice formation (Mazur, 1966). Therefore, when bacteria were stored for a long time in the ice phase, the intracellular liquid was crystallized, which could inactivate the cells.

3.4. Conclusion

It was found in this study that *E.coli* was sensitive to the freeze/thaw process, which could damage and injure cell structures and affect *E.coli* survival. The freezing temperature, storage time, cells growth phase and the preserved temperature before freezing all have effect on the viability of *E.coli* and with more freeze/thaw cycles, there would get higher *E.coli* reduction.

After the treatment using the freeze/thaw process, a portion of the *E.coli* was killed and portions of them were injured and could be recovered in a suitable environment. Using modified m-FC media by eliminating rosolic acid made it easier for the injured *E.coli* growing on the plate.

The mechanisms of freezing damage were from two factors hypothesis (Mazur, 1966): I) at low cooling rates, ice forms extracellularly and causes bacteria cells to dehydrate and shrink, and П). at high cooling rates, the intracellular liquid can be frozen. So, during the spraying freezing test, the high cooling rate can induce the intracellular ice crystal formation. With a long storage time, the spraying freezing can achieve high *E.coli* inactivation rates.

Chapter 4. Reduction of the Spores of *Bacillus megaterium* by Freeze/Thaw Process

4.1. General Biology of *Bacillus spp.*

Bacteria of the genus *Bacillus* are aerobic, endospore-forming, gram-positive rods and widely distributed in soil, air, and water. The genus *Bacillus* is taxonomically complex. Apart from the pathogenicity of *Bacillus anthracis* and *Bacillus cereus*, most other species of *Bacillus* are regarded as nonpathogenic or cause only opportunistc infections (Harwood, 1989).

The most studied *Bacillus* species belong to the mesophilic group with an optimum growth temperature between 30°C to 45°C. The metabolism of *Bacillus* adapts to a lack of continuity of nutrients in a variety of ways, most significantly by secreting various hydrolytic enzymes and by producing heat, radiation and desiccation resistant endospores that may survive in a dormant state for many years (Seaward *et al.*, 1976). The process of sporulation involves temporal and cell type-specific regulation of gene expression, intercellular communication (between mother cell and forespore), morphological differentiation and programmed cell death (Devine, 2000). The sporulation process usually takes about 6 to 8 hours under laboratory conditions of growth and the process can be divided into seven stages, as illustrated in Figure 4-1. Stage 0 to 1, the cell senses its environment and makes the decision to initiate sporulation. At stage 2, an asymmetric cell division has occurred, with the larger cell becoming the mother cell and the smaller cell is the forespore. At stage 3, the mother cell has completely engulfed the forespore to produce a cell within a cell. A cell type-specific program of gene expression has been established in each compartment at this stage. A series of morphological changes occur between stages 4 and 6 that lead to the formation of the spore cortex and spore coat. At stage 7, the mother cell lyses and releases the mature dormant spore (Doi, 1989).

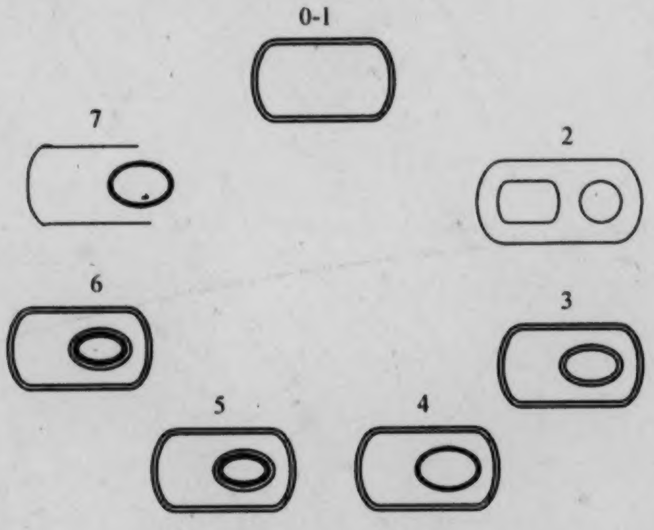


Figure 4-1. Schematic Illustration of Sporulation Stages in *Bacillus subtilus*.
(After: Doi, 1989)

Bacillus megaterium is a species of *Bacillus* that has been studied for many years because of its interesting physiology and its ability to sporulate with great efficiency. *Bacillus megaterium* and *Bacillus subtilis* both belong to 'subtilis group', which is on the basis of phenotypic similarities. These bacteria all produce acids from a range of sugars (Vary, 1993). The spore of *Bacillus megaterium* has the largest cell diameter of any aerobic spore former (1.2 to1.5 μm) and is common in soil. Megaterium means big beast (Todar, 2003). Comparing with *Bacillus subtilis*, *Bacillus megaterium* tends to produce more consistently greater amounts of spores (Guest, 2004). In this study, *Bacillus megaterium* was selected as studying bacteria.

In this section, the spores of *Bacillus megaterium* (ATCC 14581) was selected as the study microorganism to investigate how did the freeze/thaw process affect the survival of the spores and after the treatment of freeze/thaw process, how did the spores response to the effect of UV and chlorination process.

4.2. Experimental Materials and Methods

4.2.1 Materials Preparation

4.2.1.1. Buffer Solution

Batch reactor experiments were carried out using phosphate buffers and materials that were used for chlorination test would be treated as ozone demand free (ODF). Buffers at pH 6.9 and 0.05 M phosphate concentration were prepared by dissolving 2.24 g/L disodium hydrogen orthophosphate and 4.76 g/L potassium dihydrogn orthophosphate (Fisher Scientific, Nepean, Ont.) into distilled water. 1.0 N NaOH was used to adjust the pH. The buffer solution was then autoclaved at 121°C for 15 minutes and stored at 5°C.

4.2.1.2. ODF Phosphate Buffer Solution

The ODF buffers were made by bubbling ozonized gas through the prepared buffer, and stirring for 15 minutes. The solution was then stored for 1 hour. The remaining ozone was removed by boiling the buffer for 10 minutes. After cooling, using 1.0 N NaOH adjust the pH and then autoclaved at 121°C for 15 minutes. The finial ODF buffer solution was then stored in 5°C for further use.

4.2.1.3. Stock Chlorine Solution

2.0 mL 4 to 6% purified grade sodium hypochlorite (Fisher Scientific) was added into 1,000 mL sterilize phosphate buffer solution and completed mixed. DPD-colorimetric method (APHA, 1992) was used to determine the chlorine residual concentration and then left at 5°C for storage.

4.2.1.4. Sodium Thiosulphate

1.57 g sodium thiosulphate -- $Na_2S_2O_3{\cdot}5H_2O$ (Fisher scientific, N.J.) was dissolved in 100 mL distilled water and autoclaved at 121°C for 10 minutes.

4.2.1.5. Preparation of the Experimental Materials

All of the materials used in the experiments, including the plastic and glass beakers, were thoroughly cleaned in a dishwasher using Sparkleen soap (Fisher, Pittsburgh, PA) and then autoclaved at 121°C for 10 minutes.

Materials used in the chlorination test, were soaked in a about 20 mg/L ozone solution for half hour then put in oven (67 °C) for drying.

4.2.1.6. UV Apparatus

All irradiation experiments were carried out by a 10 W low-pressure collimated beam device (Ster-L-Ray Gernicidal Lamp, Model G12T6L, Atlantic Ultraviolet Corp, Haupauge, N.Y.)

4.2.2. Preparation of *Bacillus megaterium*

This spore production method was provided by Guest (2004).

Bacillus megaterium ATCC strain 14581 (American Type Culture Collection, Rockville, MD) was used as the test microorganism. Difco nutrient broth (Fisher Scientific, Itasca, IL) was used as growth culture.

4.2.2.1. Pre-Culture

The isolated *Bacillus spp.* colony were inoculated in 8.0 mL test tubes with pre-culture media (8.0 g/L Nutrient Broth, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L KCl). The culture was placed on the shaker table (180 rpm) at 37°C for 6 to 12 hours.

4.2.2.2. Spore Production

8.0 g/L nutrient broth, 0.25g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L KCl were mixed in deionized (DI) water and pH was adjusted to 8.0 and then sterilized. Stock solution of $FeSO_4$, MnCl were combined and $CaCl_2$ and added into the spore production media. The final concentration of the nutrients must be 1.0 μM $FeSO_4$, 10 μM MnCl and 1.0 mM $CaCl_2$. These three components must be sterile filtered into the spore production culture and cannot be autoclaved. The required amount of nutrient stock solution was aseptically added into the spore production media using a 0.45 μm sterile syringe filter.

The spore production flask should be aseptically inoculated with log phase growth *Bacillus spp.* culture at a dilution of approximately 1 in 1,000 by volume and put on shaker table operating at 200 rpm for 60 to 70 hours at 37 °C.

4.2.2.3. Spore Purification

After spore production, the culture was harvested and concentrated by centrifugation in a benchtop centrifuge (Model SPX; Sorvall) at 7,500 g for 20 minutes. The supernatant was removed and the pellets were re-suspended with sterile distilled (DI) water and centrifuged two more time. The pellet was then re-suspended again in sterilized DI water and heated at 100°C for 15 minutes to kill any residual vegetative cells. The spore suspension was centrifuged again and the final pellets is re-suspended in a 50% ethanol solution and refrigerated for long-term storage. Spore suspensions were examined by *Bacillus spp*. spore stain method to ensure that the preparation was comprised mainly of sporulated bacteria and not vegetative cells.

4.2.2.4. *Bacillus megaterium* Spore Stain

The method used to stain spores was the Schaeffer-Fulton Stain. It employs hot 5% malachite as the intense stain and 1% safranin as a conterstain. Thus the spores attains green, the vegetative cells light red (Guest, 2004).

A clean slide was dried and placed on an O-ring ¾ way up the retort stand. A smear of the culture was placed on the slide and flooded with 5% aqueous malachite green for approximate 1 minute, during which time the slide was heated to steaming 3 to 4 times. The excess stain was washed off with DI water. The counterstain was obtained with 1% safranin for 30 minutes, then washed off in water. The stained spores were air dried and examined by microscopy (100 × magnification).

4.2.3. *Bacillus megaterium* Enumeration

In this experiment, pour plate method was used for enumeration of *Bacillus megaterium*.

4.2.3.1 Preparation of the Experimental Medium

Sufficient number of molten nutrient agar tubes were prepared, which were comprised with 8.0 g/L Difco nutrient broth (Fisher Scientific, Itasca, IL) and 16 g/L granulated agar (Fisher Scientific, Itasca, IL). These agar tubes were autoclaved at 121°C for 20 minutes and then placed in 50°C water bath for further use.

Dilution water was prepared by autoclaving distilled (DI) water at 121°C for 20 minutes. 9.0 mL of autoclaved dilution water was then aseptically transferred into sterilized tubes.

4.2.3.2. Procedure for Performing the Pour Plates Method

Firstly, the sample was thoroughly mixed. 1.0 mL of sample was aseptically pipetted and transferred into the first Falcon tube containing 9.0 mL of sterile water. The suspension was vortex mixed and the serial dilutions were completed. 1.0 mL of desired dilution was aseptically transferred to a sterile 90 mm Petri dish by raising the lid and injecting the sample and placed the lid back on the Petri plate. The prepared nutrient agar tubes were then taken from the water bath and the outside water was cleaned. The molten agar was aseptically poured over the sample. The Petri plates were placed in the laminar flow hood with the lid partially removed for 3 to 5 minutes for the agar to solidify. The plates were inverted and incubated at 37 °C for 3 days and then count the colonies in the plate.

4.2.4. Experiments

4.2.4.1. Batch Test for *Bacillus megaterium* Freezing

The objective of the batch test was to determine how the freeze/thaw process could affect the spores of *Bacillus megaterium* survival.

Bacteria stock solution was transferred into the sterilized phosphate buffer solution and mixed for10 minutes. The final concentration of *Bacillus megaterium* was between 10^5 to 10^6 CFU/mL. A 50 mL mixed solution was put in 100 mL plastic beakers and covered with sterilized aluminum foil, then placed in the freezer. After the solution was frozen, the sample was melted at 5°C. The density of the surviving microorganism was then enumerated.

4.2.4.2. Spraying Freezing Test for *Bacillus megaterium*

The procedure was the same as in the *E.coli* test. The detailed information can refer to 3.2.5.3 in chapter 3.

The objective of the spraying test was to study whether the freezing rate and freezing behavior had significant effects on the spores of *Bacillus megaterium* deduction and compare the results with those obtained from batch tests.

4.2.4.3. Chlorination Test for *Bacillus megaterium* Reduction

The objective of chlorination test was to determine whether the freeze/thaw process had effects on the biological character of spores and did the process make the spores becoming more sensitive to the chemical disinfectant.

Water samples were prepared by diluting the stock *Bacillus* solution in sterilized ODF phosphate buffer solution. The final concentration of *Bacillus megaterium* was between 10^5 to 10^6 CFU/mL. After that, a 75 mL water sample was placed in a 200 mL flask and gently mixed with a Teflon®- magnetic stir bar and then a pre-determined amount chlorine stock solution was added in the flask. In the designed time interval, 1.0 mL water sample was taken from the flask into a test tube, which contained 8.0 mL sterilized DI water and 0.5 mL sterilize 0.1% sodium thiosulfate. The contents of tube was then mixed vigorously on a Maxi-Mix vortex mixer (Thermolyne Co., Dubuque, Ia.). Pour plates method was used to determine microorganism concentration. Throughout the contact period, the microorganism suspension was continuously stirred by a Teflon®- magnetic stir bar. DPD-colorimetric method (APHA, 1998) was used to determine the chlorine residual concentration in water solution.

4.2.4.4. UV Test for *Bacillus megaterium* Reduction

The objective of UV test was to exam how did *Bacillus megaterium* respond the UV light radiation after the freeze/thaw process.

A spectrophotometer-- Ultrospec® 3000 (Pharmacia Biotech, England) was used in this experiment to determine the absorbance of water sample at 254 nm wavelengh. Germicidal UV dose for a low-pressure lamp was determined by the method provided by Jim Bolton (Bolton Photosciences Inc. 2002). Six factors were considered, Petri factor, reflection factor, sensor factor, germicidal factor, water factor and divergence factor.

A petri dish (60 mm diameter × 15 mm height) was placed on a stir plate. 20 mL sample solution was transferred into the Petri dish and the suspension was gently and constantly stirred during the exposure period by a magnetic bar. In the appropriate time, 1.0 mL sample was taken from the dish into a test tube contained 9.0 mL sterilized DI water. The contents of tube was then mixed vigorously on a Maxi-Mix vortex mixer (Thermolyne Co., Dubuque, Ia.). Then pour plates method was used to determine the microorganism concentration.

4.2.4.5. Scanning Electron Microscope Test

The concentration of the spores of *Bacillus megaterium* solution for SEM test was set at 10^5 to 10^6 CFU/mL. The images of the spores of *Bacillus megaterium* in liquid phase were conducted by Dr. Ming Chen and the images of the spores of *Bacillus megaterium* in ice phase was taken by Mr. George Braybrook at University of Alberta.

All the procedures for SEM test are the same as in the *E.coli* test, which can refer to 3.2.5.5.

4.3. Results and Discussion

4.3.1. The Effect of Freeze/Thaw Process on *Bacillus megaterium*

Experimental results obtained from this study indicated that the freezing at -15°C had no significant effect the viability of the spores of *Bacillus megaterium*. The results are reported in Table A-20 of Appendix A. Using t-test to check results, there are no significant reduction to the spores after freezing. The data analysis is listed in Table 4-1.

Table 4-1. t-test for *Bacillus megaterium*. Reduction

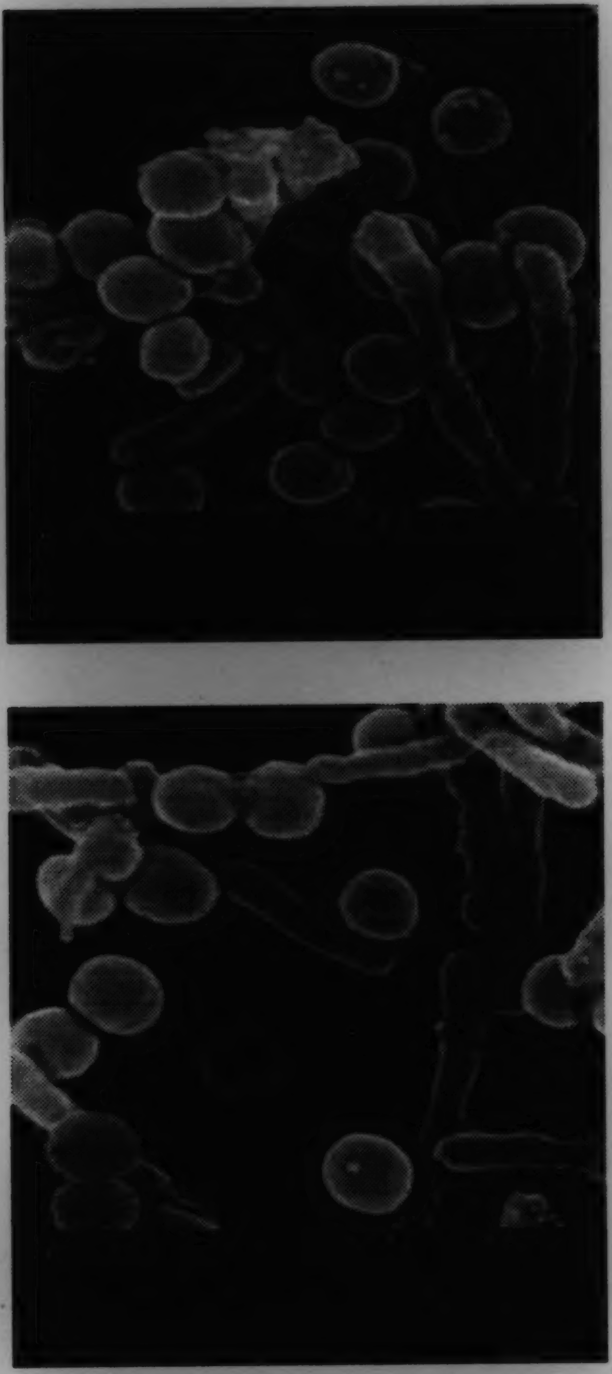
Average Removal	0.005 log units
Standard deviation	0.0247
Assume removal is	0
$t_{(0.025,9)}$	2.262
t	0.649
$t < t_{(0.025,9)}$, accept assumption	

Because $t < t_{(0.025,9)}$, it suggested that the freeze/thaw process had no significant effect on the inactivation of *Bacillus megaterium* spores.

Image 4-1 shows the *Bacillus megaterium* spores without freezing. Image 4-2 presents the *Bacillus megaterium* spores after freezing. Image 4-3 is the *Bacillus megaterium* spores in the ice structure.

From the images of the *Bacillus megaterium*, there were no changes in the shape of the structure of spores after freezing. The images also showed that the *Bacillus megaterium* spores were clamped in the ice.

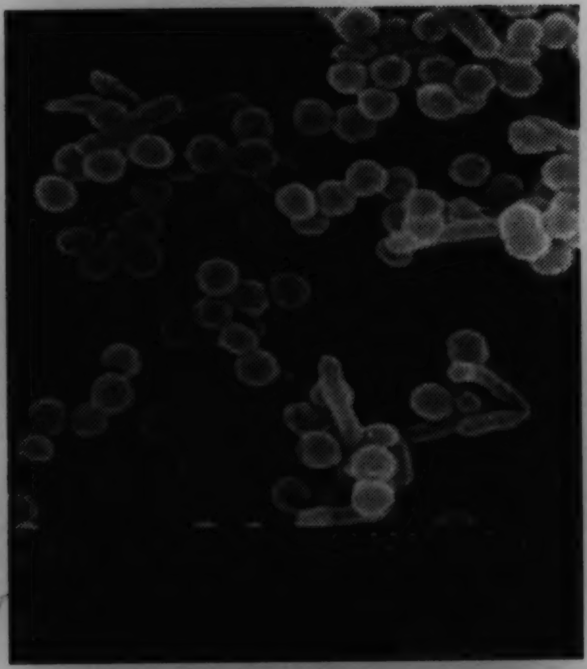
Previous studies by Lund et al. (2000) and Doyle et al. (1997) demonstrated that the quick or slow freezing of spores in buffer or in pea juice (pH = 7.1) had no effect on viability and repeated freezing and thawing had not much more effect to their survival. The experimental results of this study confirm that the spores are much more resistant than vegetative cell to freeze/thawing process.



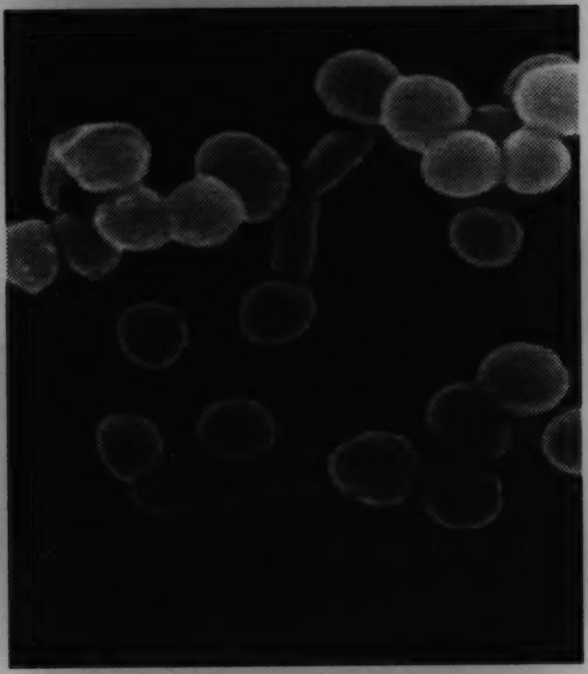
(20,000 ×)

(20,000 ×)

Image 4-1. ***Bacillus megaterium*** **(spores) Before Freezing**



(10,000 ×)



(20,000 ×)

Image 4-2. ***Bacillus megaterium*** **(spores) After Freezing**

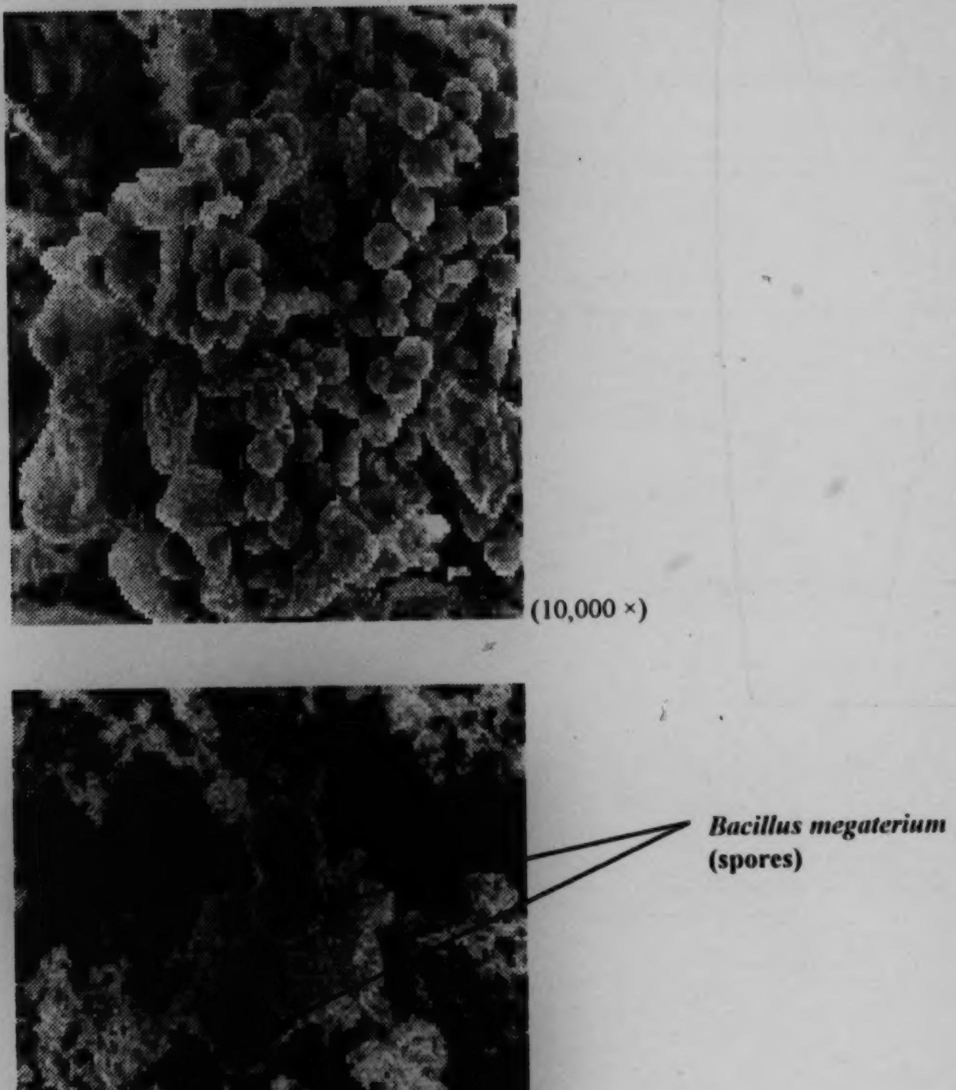

(10,000 ×)
Bacillus megaterium
(spores)
(17,000 ×)

Image 4-3. ***Bacillus megaterium*** **(spores) in the Ice**

4.3.2. Spraying Freezing Test for *Bacillus megaterium* Reduction

The spraying freezing process had no significant effect on the viability of spores, although the freezing rate was higher than that of the batch tests. The experimental results were listed in Table A-21 of Appendix A and the t-test for data analysis is shown in Table 4-2.

Table 4-2. t-test for *Bacillus megaterium* Reduction in Spraying Test

	Without storage		Storage 2days
Average Reduction	0.018		0.020
Standard deviation	0.075		0.037
Assume reduction	0		0
$t_{(0.05,3)}$	2.353	$t_{(0.05,4)}$ =	2.132
t	0.487	t	1.243
	Accept assumption		

As indicated in Table 4-2, the storage time also had no influence on the survival of *Bacillus megaterium*.

4.3.3. Freezing Combined with UV Test for *Bacillus megaterium* Reduction

Ultraviolet (UV) disinfection is now considered an acceptable process for inactivation of microorganisms in water treatment. The germicidal effects of UV light involve photochemical damage to RNA and DNA within the cells of an organism (Darby *et al.*, 1995). Absorption of UV light in the range of 200 to 300 nm damages the thymine nucleotides in the DNA strand, causing to form dimmers. If a high enough degree of themine dimerization is achieved, the DNA cannot be properly copied and organism is "inactivated" (Mackey et al., 2001).

Previous studies had proved that UV was an effective method to inactivate spores and some other bacteria, which were resistant to chlorine disinfection. For *Bcaillus subtilus* spores ATCC 6633, a 3-log reduction could be achieved at UV dose from 58 to 63 mJ/cm^2 (Chang *et al.*, 1985, Taylor, 2003, Gravetz and Linden, 2004).

In this experiment, it had worked and tried to find whether the freeze/thaw process could affect the spores and if the process made them becoming more sensitive or resistant to UV effects. The average reductions under in different UV fluences are shown in Table 4-3 and Figure 4-2.

Table 4-3. UV Light for *Bacillus megaterium* Inactivation

	Reduction without freezing		Reduction after freezing	
Fluence	Ave. Reduction	Stdev.	Ave. Reduction	Stdev.
mJ/cm^2	(-log(N/N$_0$)		(-log(N/N$_0$)	
10.25	0.666		0.718	
20	1.069	0.060	1.137	0.237
40	2.910	0.236	2.725	0.188
60	3.947	0.278	3.100	0.195
80	4.589	0.275	3.418	0.241

At the low UV fluence, 10.25 to 40 mJ/cm^2, there were no statistical differences for the *Bacillus* inactivation. At the later phase of UV irradiation, 60 to 80 mJ/cm^2, the freezing process seemed to make the cells more resistant to the UV. The results of t-test are reported in Appendix C.

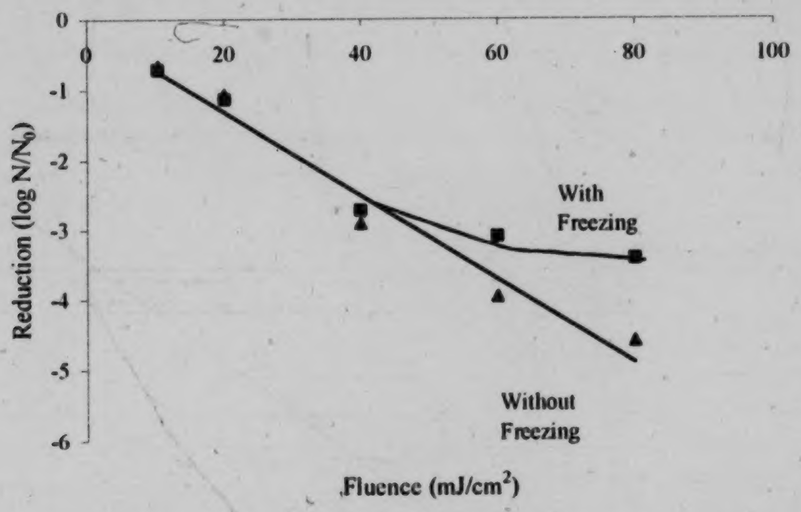


Figure 4-2. Comparison of *Bacillus spp.* Reduction with or without Freezing

Figure 4-2 shows the reduction curves of *Bacillus megaterium* under different UV doses. With freezing, the removal curve becomes a biphasic curve with a "tail". Two kinds of conception were proposed to explain the tailing phenomenon: 1) the vitalistic conception, 2) the mechanistic conception (Cerf, 1977).

The Vitalistic Conception

The tenet of vitalism is that the character of bacteria, such as the resistance to a lethal agent or process, may be possessed to different degrees and between the various individual microorganisms of a pure culture, the difference in the degree of resistance is permanent. This has led to the postulate that survival times should be normally distributed, but this assumption had never been proved (Cerf, 1977).

The Mechanistic Conception

According to Lee & Gillert (1918), 1) the spores have a general similarity of resistance between the different individuals of a population; 2) the process of destruction is 'an orderly time process presenting a close analogy to a chemical reaction; 3) therefore, at any given time only a proportion of molecules of the interacting substances are in a position or condition to participate in the inactivation reaction; and 4) consequently, the destruction process may be regarded as a reaction of a first order or higher order than runs to completion by a series of unimolecular reactions and fitting the logarithmic order of death.

From above conceptions, one of the explanations for this phenomenon is the mechanism of resistance. Alderton et al. (1964) had shown that spores devoid of exchangeable calcium (H-form), would give a concave upward survival curve when heated in a medium rich in calcium, and that, in such conditions, they could regain the heat resistance of the native spores. Han (1975) had referred to a modification of resistance of spores during the treatment. According to Prokop & Humphrey (1970), this modification 'could be thought of as mutation occurring during the process'. During the freezing, the individual spore in the population developed a different degree of resistance because of adaptation to cooling and then the acquired cooling resistance cells become more resistant than other cells.

Another factor that may be involved is the water activity. Murrell & Scott (1966) found, for *Bacillus megaterium*, the heat resistance increased steadily with decreasing water activity (a_w) values and at a water activity 0.3 to 0.4, spores could reach the maximum resistant and gave rise to concave upward survival cures. In the freezing, the water activity was decreasing as the temperature dropped. Therefore, during the freezing process, the spores were exposed to lower water activity solution and then adapted to more resistance.

The tailing may also be caused by clumping, which can be formed in the freezing process. During the freezing process, the spores can be concentrated and then make them clumping together and then become more resistant to adverse environments.

The exact mechanism for the tailing of freezing curve is not fully understood. That needs further investigation and study.

4.3.4. Chlorination for *Bacillus megaterium* Reduction

Chlorination is the most common method for inactivating microorganism. It is a well known fact that chlorine precipitates proteins. It is believed that chlorine can alter the chemical arrangement of enzymes and inactivate them directly and also can destroy the selective permeability of the cells wall membrane and thus allow vital solutes and nutrients to diffuse out of the cells. Another proposed mechanism for chlorine disinfection is that compounds of chlorine hydrolyze the cell wall polysaccharides thus weakening the cell wall (Darby *et al.*,1995).

In this experiment, combined two processes—chlorination and freezing/thawing to test how the freezing affect the spores of *Bacillus megaterium* survival. From the experimental results, after freezing, the different chlorine concentration had the different effect to the survival of spores. Table 4-4 and Figure 4-3 to Figure 4-5 compare the responses of spores-- with and without freezing in different chlorine concentrations.

Table 4-4. Chlorination for *Bacillus megaterium* Reduction

Without freezing			With Freezing		
Contact Time (minutes)	Reduction ($-\log N/N_0$)	Stdev*.	Contact Time (minutes)	Reduction ($-\log N/N_0$)	Stdev*.
Free Chlorine: 2.0mg Cl_2/L					
5	0.192	0.052	5	0.062	0.010
10	0.305	0.073	10	0.222	0.086
20	0.358	0.082	20	0.391	0.172
40	0.459	0.179	40	0.372	0.119
Free Chlorine: 4.0mg Cl_2/L					
5	0.371	0.100	5	0.309	0.091
10	0.756	0.229	10	0.606	0.201
20	1.950	0.527	20	1.704	0.473
40	3.244	0.527	40	3.491	0.381
Free Chlorine: 6.0mg Cl_2/L					
2.5	0.365	0.092	2.5	0.316	0.011
5	0.594	0.290	5	0.565	0.316
10	1.513	0.316	10	1.899	0.591
20	3.404	0.585	20	4.427	0.615

* Standard Deviation

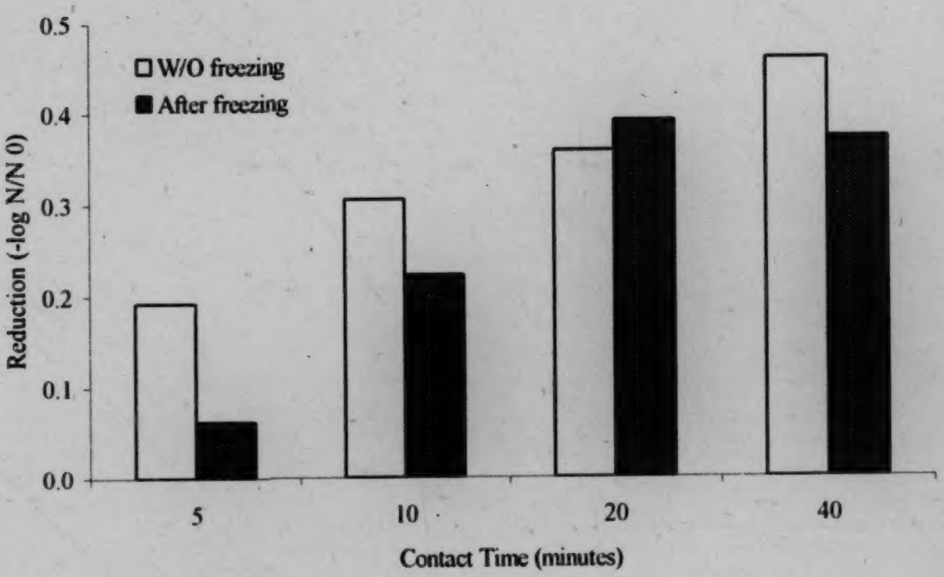


Figure 4-3. Chlorination (2.0 mg Cl_2/L) for *Bacillus megaterium* Reduction

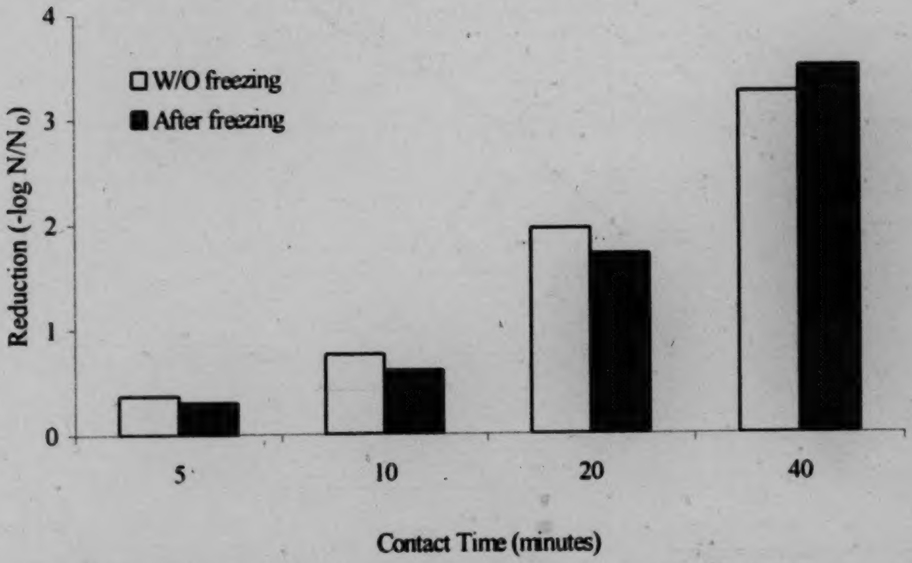


Figure 4-4. Chlorination (4.0 mg Cl_2/L) for *Bacillus megaterium* Reduction

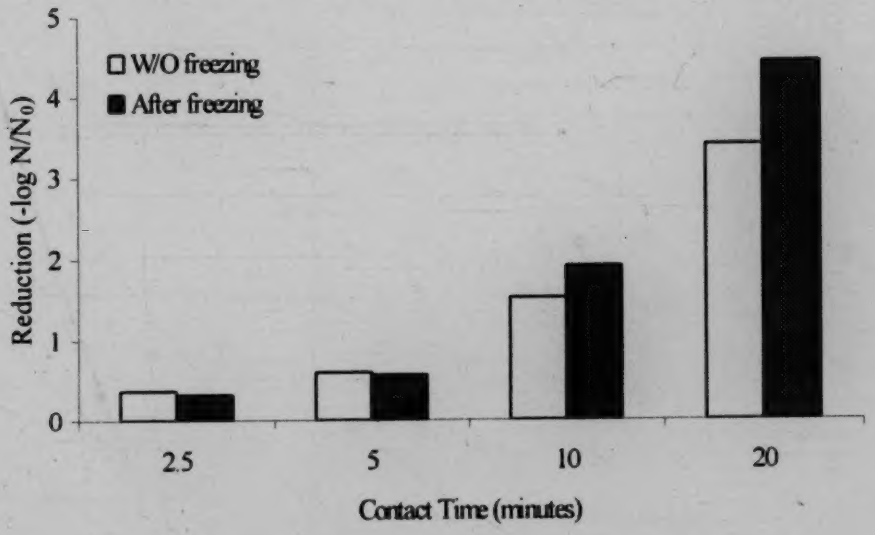


Figure 4-5. Chlorination (6.0 mg Cl_2/L) for *Bacillus megaterium* Reduction

The experimental results showed that at 4.0 mg Cl_2/L, there were no differences in terms of inactivation of *Bacillus megaterium* either with freezing or without freezing. When the free chlorine concentration was 2.0 mg Cl_2/L, at the beginning, the freezing appeared to made the spores more resistant to the chlorine. At 6.0 mg Cl_2/L, after freezing, the reduction rate of spores expose to chlorine became higher than the control samples (without freezing). This suggested that the freeze/thaw process damaged the cells' structure but the damage was not enough to affect the vitality. However, if exposed to the chlorine, these cells were more easily inactivated. At the same time, after freezing, the spores can be concentrated and then make them clumping together. Accounting to Stumbo (1973), if clumping occurs, first it can account for a tailing off and secondly a decrease of survivors will be observed. At the low chlorine concentration (2.0 mg Cl_2/L), the spores presented higher resistance than the control samples.

So, after freeze/thaw process, the cells structure had suffered non-lethal damage but at the same time, the clumping also made the cells becoming more resistant to the effect of chlorine. When the chlorine concentration was low, the clumping phenomenon can protect the cells from the germicidal effects and while the chlorine concentration became higher, the clumping will be not enough to protect them. Therefore, the damaged cells became more sensitive to the effect of chlorination.

4.5. Conclusion

The process of freeze/thaw process has no significant effect on the spore of *Bacillus megaterium* reduction. But it could change the water activity (a_w), concentrate cells and form clumping, which may damage spore structure and then affect its ability to resist the chemical or physical attack. The exact mechanisms by which freezing affect the resistance of spores are not fully clear.

Chapter 5. Summaries and Recommendations

5.1. Summaries

From the study of the freeze/thaw process for microorganisms inactivation in buffer solution, the following conclusions have been reached:

1. For *E.coli*, the holding time in the freezing temperature, the salt concentration and the preserved temperature of bacteria before freezing had significant effect on the survival of *E.coli*. Longer storage time, higher salt concentration and lower preserved temperature reduced the viability of *E.coli*.
2. Freezing temperature had very important effect on the *E.coli* survival. There was an optimum temperature for *E.coli* survival. Too high or too low a temperature could reduce *E.coli* survival. In this study, the *E.coli* reduction rate at different temperature was: -5°C > -35°C >-15°C.
3. The effect of freezing on the viability of *E.coli* was different at different cell growth phases. In the stationary growth phase, *E.coli* were most resistant to freezing and on the other hand, at the log growth phase, *E.coli* were the most sensitive to the freezing.
4. The freeze/thaw process can damage the cell structure and make the cell shrink, leak and then lose its viability. Some of the damaged cells could be recovered in a suitable environment.
5. The freeze/thaw cycle could further inactivate the damaged cells and reduce the survival rates.

6. The spraying freezing, which usually had a higher and non-constant freezing rate, could nucleate the internal cellular water, and if the cells were held in storage for a longer period in the freezing temperature, that would cause very high lethal consequences.
7. For *Bacillus megaterium* spores, the freezing/thawing process, including spraying freezing, had no lethal effect to their survival.
8. With the freezing treatment, *Bacillus megaterium* spores become more resistant to certain doses of UV radiation and more sensitive to high chlorine concentrations.
9. Freeze/thaw can change or damage spore structure, change the water activity and clump the bacteria and then affect the ability of resistance to the adverse environment.

5.2. Potential Use of the Freeze/thaw Process

Snowmaking is one of the potential uses for this technique to treat wastewater. Delta Engineering (Ottawa, Canada) has used a snow making process to treat wastewater and many of the contaminants and microorganisms could be concentrated and removed (Delta Engineering, 2001). There are a number of beneficial attributes to the use of snowmaking as a means of effluent disposal. For example, if an effluent were applied as snow onto agricultural or forested land during the winter months, the majority of the nutrients present in the snowpack would be concentrated into the early portion of the snowmelt. The soil would be saturated with this nutrient-rich concentrate, leaving the remaining, relatively pure melt water as surface runoff (Rabinowita *et al.*, 1988).

Another potential use for this process is in the lagoon study. In the wintertime, lagoons are always frozen and stop dispose effluent. Through the study of the freeze/thaw process, we can get better understanding of the perform of the lagoon in the cold weather. Such as how many microorganisms will be gotten reduction, how many solutes or pollutants will be left in the bottom and how many in the runoff.

5.3. Recommendations

1. Further studies are necessary to understand the mechanisms of freezing on the microorganisms viability.
2. It would be useful to study how the freeze/thaw process affects other kind of microorganisms, such as algae, protozoa, and virus particles.
3. It is also important to study the methods for practical application.

References

Adams, M.R. and Moss, M.O. 2000. Food microbiology, 2^{th} edition. The Royal Society of Chemistry, UK.

Alderton, G.; Chen, J.K. and Ito, K.A. 1964. Effect of lysozyme on the heated resistance of *Clostridium botulinum* spores. *Applied Microbiology*, vol. 27, pp. 797-901.

American Public Health Association (APHA). 1998. Standard methods for the examination of water and wastewater, 20^{th} edition. American Public Health Association, Washington, D.C.

ATCC, (American Type Culture Collection). 2004. Bacteriology FAQ. http://www.atcc. org /TechnicalInfo/faqBacteriology.cfm

Baker, R.A. 1967. Trace organic contaminant concentration by freezing-I. Low inorganic aqueous solution. *Water Research*, vol. 1, pp.61-77.

Bretz, H.W. and Kocka, F.E. 1967. Resistance to actinomycin D of *Escherichia coli* after frozen storage. *Canadian Journal Microbiology*, vol.13, pp.914-923.

Calcott, P.H. and Macleod, R.A. 1974. Survival of *Escherichia coli* from freeze-thaw damage: Influence of nutritional status and growth rate. *Canadian Journal Microbiology*, vol.20, pp. 683-689.

Calcott, P.H. and Macleod, R.A. 1975. The survival of *Escherichia coli* from freeze-thaw damage: permeability barrier damage and viability. *Canadian Journal Microbiology*, vol.21, pp.1724-1732.

Calcott, P.H.; Lee, S.K. and Macleod, R.A. 1976. The effect of cooling and warming rates on the survival of a variety of bacteria. *Canadian Journal Microbiology*, vol.22, pp. 106-109.

Calcott, H. P. 1978. Freezing and thawing microbes. Meadowfield Press Ltd., Bushery, England.

Cerf, O. 1977. A review: Tailing of survival curves of bacterial spores. *Journal of Applied Bacteriology*, vol.42, pp.1-19.

Chalmers, B. Principles of solidification. New York, Wiley, 1964.

Chang, J.C.H.; Ossof, S.F.; Lobe, D.C.; Dorfman, M.H.; Dumais, C.M.; Qualls, R.G. and Johnson, J.D. 1985. UV inactivation of pathogenic and indicator microorganisms. *Applied Environmental Microbiology*, vol.49, pp.1361-1365

Chu, C.P.; Feng, W.C.; Chang, B.V.; Chou, C.H. and Lee, D.J. 1999. Reduction of microbial density level in wastewater activated sludge via freezing and thawing. *Water Research*, vol.33, No.16, pp 3532-3535.

Columbia University Press, 2001. The columbia encyclopedia, 6th edition. Columbia University Press.

Darby, J.; Heath, M.; Jacangelo, J.; Loge, F.; Swaim, P. and Tchobanoglous, G. 1995. Comparison of UV irradiation to chlorination: Guidance for achieving optimal UV performance. Project 91-WWD-1, Water Environmental Research Foundation.

Davies, J. E. 1970. The frozen cell. Churchill, London.

Davies, R. and Obafemi, A. 1985. Response of micro-organisms to freeze-thaw stress. *In*: Microbiology of frozen foods. Elsevier Applied Science Publishers, London.

Delta Engineering Ltd. 2001. Freezing cleans food processing wastewater, summary report. Alberta Agriculture, Food and Rural Development, AB, Canada.

Devine, M. K. 2000. *Bacillus subtilis*, genetics. *In*: Encyclopedia of microbiology, vol.1, 2nd. Academic Press.

Doi, H. R. 1989. Sporulation and germination. *In*: Biotechnology handbooks 2, *Bacillus*. Plenum Press, New York and London.

Doyle, P.M.; Beuchat, R.L.; and Montville, J.T. 1997. Food microbiology—fundamentals and frontiers. American Society for Microbiology, Washington D.C.

Finch R.G.; Stiles E.M. and Smith W.D. 1987. Recovery of a marker strain of *Escherichia coli* from ozonated water by membrane filtration. *Applied and Environmental Microbiology*, Dec. 1987, p.2894-2896.

Food Science Australia. 2004. Water activity. http://www.foodscience.afisc.csiro.au/water_fs.htm.

Frazier, C. W. and Westhoff, C. D. 1988. Food microbiology, 4th Edition. McGraw-Hill, Inc., U.S.A.

Gabis, D.A. 1970. Studies on the effects of low temperature storage on the metabolism of *Escherichia coli*. PhD. Thesis, North Carolina State University.

Gao, W. 1998. Spray freezing as a treatment alternative for industrial wastewater treatment. Ph.D. Thesis, University of Alberta, Edmonton, Alberta.

Gay, G.; Lorain, O.; Azouni, A. and Aurelle, Y. 2003. Wastewater treatment by radial freezing with stirring effects. *Water Research*, vol. 37, pp. 2520-2524.

Gelinas, P.; Fiset, G.; LeDuy, G.A. and Goulet, J. 1989. Effect of growth conditions and trehalose content on cryotolerance of bakers' yeast in frozen doughs. *Applied Environmental Microbiology*, vol.55, pp2453-2459.

Gravetz, M. H. and Linden, G. K. 2004. UV disinfection of indigenous aerobic spores: Implications for UV reactor validation in unfiltered waters. *Water Research*, vol.38, pp. 2898-2906.

Guest, R.K. 2004. *Bacillus spp.* spore production—a short guidance manual. University of Alberta, Edmonton, Canada.

Haas, C.N. 1986. Wastewater disinfection and infectious disease risks. Environmental Control. Vol.17, pp.1-20.

Halde, R. 1980. Concentration of impurities by progressive freezing. *Water Research*, vol. 14, pp.575-580.

Han, Y.W. 1975. Death rates of bacterial spores: nonlinear survivor curves. *Canadian Journal of Microbiology*, vol. 21, pp. 1464-1467.

Harwood, R. C. 1989. Biotechnology handbooks 2, *Bacillus*. Plenum Press, New York and London.

Hrudey, E.S. and Hrudey, J.E. 2004. Safe drinking water -- lessons from recent outbreaks in affluent nations. IWA Publishing, London.

Ingram M. and Mackey, B.M. 1976. Inhibition and inactivation of vegetative microbes. Academic Press, London.

Jim Bolton-Bolton Photosciences Inc., 2002. Germicidal fluence (UV dose) calculations for a low pressure UV lamp. Edmonton, Canada.

Karl, D.M., Bird, D.F., Bjorkman, K., Houlihan, T., Shackelford, and Tupas, L. 1999. Microorganisms in the Accreted Ice of Lake Vostok, Antarctica. *Science*, 286: pp. 2144-2147.

Lindeberg, G. and Lode, A. 1963. Release of ultraviolet-absorbing material from *Escherichia coli* at subzero temperature. *Canadian Journal Microbiology*, vol.9, pp.523-530.

Lorain, O.; Thiebaud, P.; Badorc, E. and Aurelle, Y. 2001. Potential of freezing in wastewater treatment: Soluble pollutant applications. *Water Research*, vol. 35, No.2, pp.541-547.

Lund, M. B.; Baird-Parker, C. T. and Gould, W. G. 2000. The microbiological safety and quality of food. Aspen Publishers, Inc., Maryland.

Mackey, B.M. 1984. Lethal and sublethal effects of refrigeration, freezing and freezing-drying on microorganisms. *In*: The revival of injured microbes. Academic Press, Orlando, Florida.

Mackey, D. E.; Cushing, S. R. and Crozes, F. G. 2001. Practical aspects of UV disinfection. AWWA Research Foundation, Denver, CO.

Macleod, R. A. and Calcott, H. P. 1976. Cold shock and freezing damage to microbes. *In*: Survival of vegetative bacteria. Cambridge University Press, Cambridge.

Macleod, R.A.; Kuo, S.C. and Gelinas, R. 1967. Metabolic injury to bacteria. П. metabolic injury induced by distilled water or Cu^{2+} in the plating diluent. *Journal of Bacteriology*, vol. 93, pp. 961-969.

Martel, C. J. 2000. Influence of dissolved solids on the mechanisms of freee-thaw conditioning. *Water Research*. vol.34, pp.657.

Martel, C.J. and Diener, C.J. 1991. A pilot scale study of alum sludge dewaterability in a freezing bed. *Journal of AWWA*, vol.83, pp.51.

Mazur, P. 1966. Physical and chemical basis of injury in single-celled microorganisms subjected to freezing and thawing. *In:* Cryobiology. Academic Press, New York.

McCarron, J.E. 1965. Survival and distribution of mesophilic bacteria in ice. M.Sc. thesis, Colorado State University, Ford Collins, Colorado.

Meryman, H.T. (1966). Cryobiology, Academic Press, New York.

Metcalf & Eddy, Inc. 2003. Wastewater engineering—treatment and reuse. 4th edition. The McGraw-Hill Companies, Inc., U.S.A.

Minor, T.E. and Marth, E.H. 1972. Lose of viability by staphylococcus aureus in acidified media. *J. Milk Food Technology*. Vol.35, pp. 548-555.

Montgomery, C.D. 2001. Design and analysis of experiments, 5th. John Wiley & Sons, Inc., USA.

Morichi, T. 1969. Freezing and drying of microorganisms. University of Tokyo Press, Tokyo.

Murrell, W.G. and Scott, W.J. 1966. The heat resistance of bacterial spores at various water activities. *Journal of General Microbiology*, vol. 43, pp. 411-425.

Ormeci, B. and Vesilind, A. P. 2001. Effect of dissoled organic material and cations on freeze-thaw conditioning of activated and alum sludges. *Water Research*. vol. 35, pp. 4299-4306.

Parker, V. L. and Martel, C. J. 2002. Long-term survival of enteric microorganisms in frozen wastewater. U.S. Army Engineer Research and Development Center, Cold Regions Research and Engineering laboratory, ERDC/CRREL Report TR-02-16.

Parker, V. L.; Yushak, L. M. and Martel, C. J. 2000. Bacterial survival in snow made from wastewater. U.S. Army Engineer Research and Development Center, Cold Regions Research and Engineering laboratory, ERDC/CRREL Report TR-00-9.

Partyka, V. 1986. Freezing for wastewater recovery. *Metal Finishing*, 84 (11), pp. 55-57.

Patti W. 2004. Determination of water activity using the Decagon AquaLab CX-2 and series 3. *In*: Food program. http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/volume_3/e_mflp66e01.html.

Porkop, A. and Humphrey, A.E. 1970. Kinetics of disinfection. *In*: Disinfection. Marcel Dekker, New York.

Presswood, G. W. and Strong, K. D. 1978. Modification of M-FC medium by eliminating rosolic acid. *Applied and Environmental Microbiology*, July, pp.90-94.

Rabinowita, B. Vassos, T.D. and Hyslop, W.F. 1988. The use of snowmaking for wastewater treatment and disposal. In: Proceedings of Environment Canada et al. Wastewater Treatment 11th International Symposium, Montreal, 1988: 1-13.

Rabinowita, B.; Vassos, T.D.; Hyslop, W.F.; ZapfGilje, R. and Mavinic, D.S. 1988. Secondary effluent disposal through snowmaking. In: Proceedings, Joint CSCE-ASCE National Conference on Environmental Engineering. Vancouver, B.C., pp 763-744.

Ray, B. 1989. Enumeration of injured indicator bacteria from foods. *In:* Injured index and pathogenic bacteria: occurrence and detection in foods, water and feeds. CRC Press, Boca Raton, Florida.

Ray, B.; Janssen, D.W. and Busta, F.I. 1972. Characterization of the repair of injury induced by freezing *Salmonella anatum*. *Applied Microbiology*, Vol. 23, pp. 803-809.

Ray, B., and Speck, M.L. 1973. Freeze-injury in bacteria. CRC Critical Reviews, *In*: *Clinical Laboratory Science*, No.4, pp.161.

Ray, B.; Speck, M.L. and Dobrogosz, W.J. 1976. Cell wall lipopolysaccharide damage in *Escherichia coli* due to freezing. *Cryobiology*, vol.13, pp.153-160.

Robinson, R. K. 1985. Microbiology of frozen foods. Elsevier Applied Science Publishers, London.

Sanin, F. D.; Vesilind, P. A. and Martel, C. J. 1994. Pathogen reduction capabilities of freezing/thawing sludge conditioning. *Water Research*, vol.11, pp. 2393-2398.

Schaechter, M. 2000. *Escherichia coli*, general biology. Encyclopedia of Microbiology. 2nd Edition. Academic Press, New York.

Seaward, M. S.; Cross, T.; and Unsworth, B. A. 1976. Vialbe bacterial spores recovered from archaeological excavation. *Nature*, vol.261: pp.407-408.

Shapiro, J. 1961. Freezing out, A safe technique for concentration of dilute solutions. *Science N.Y.* vol.133, pp. 2063-2064.

Silvares, O.M.; Cravalho, E.G; Toscano, W.M. and Huggins, C.E. (1975). The thermodynamics of water transport from biological cells during freezing. *J. Heat Transfer*, vol.97, pp.582-590.

Stumbo, C.R. 1973. Thermobacteriology in food processing, 2nd. Academic Press, New York.

Taylor, M. 2003. UV Disinfection of drinking water-draft for discussion. Ministry of Health New Zealand.

Todar, K. 2003. The genus *Bacillus*. University of Wisconsin-Madison. USA.

USEPA, (U.S. Environmental Protection Agency). 2004. *E. coli* O157:H7 in drinking water. http://www.epa.gov/safewater/ecoli.html.

Van den berg, L. 1968. Low temperature biology of foodstuffs. Pergammon Press, London.

Vary, S. P. 1993. The genetic map of *Bacillus megaterium*. In: *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington D.C.

Appendix A. Raw Data of Experiment

1. *E.coli* Test

1.1. 2^{5-1} Factional Factorial Design

Table A-1. 2^{5-1} Factional Factorial Design (m-FC method)

	Replicate 1			Replicate 2			Replicate 3			
Run	Primary solution	After freezing	Reduction	Primary solution	After freezing	Reduction	Primary solution	After freezing	Reduction	Average Reduction
	(CFU/mL)	(CFU/mL)	-log N/N$_0$	(CFU/mL)	(CFU/mL)	-log N/N$_0$	(CFU/mL)	(CFU/mL)	-log N/N$_0$	-log N/N$_0$
1	3.70E+07	7.40E+06	0.70	1.95E+07	8.60E+06	0.36	3.00E+07	6.60E+06	0.66	0.57
2	3.70E+07	8.80E+06	0.62	1.95E+07	3.10E+06	0.80	3.00E+07	2.80E+06	1.03	0.82
3	3.70E+07	9.00E+05	1.61	1.95E+07	1.25E+06	1.19	3.00E+07	2.20E+06	1.13	1.31
4	3.70E+07	4.30E+06	0.93	1.95E+07	1.80E+06	1.03	3.00E+07	1.63E+06	1.26	1.08
5	8.50E+06	2.37E+06	0.55	1.39E+07	1.30E+06	1.03	1.20E+07	4.80E+06	0.40	0.66
6	8.50E+06	3.30E+06	0.41	1.39E+07	3.10E+06	0.65	1.20E+07	4.20E+06	0.46	0.51
7	8.50E+06	5.50E+05	1.19	1.39E+07	4.00E+04	2.54	1.20E+07	2.20E+05	1.74	1.82
8	8.50E+06	5.00E+04	2.23	1.39E+07	4.70E+04	2.47	1.20E+07	1.46E+05	1.91	2.21
9	2.90E+07	1.20E+07	0.38	1.90E+07	1.03E+07	0.27	2.55E+06	1.28E+06	0.30	0.32
10	2.90E+07	1.35E+07	0.33	1.90E+07	9.50E+06	0.30	2.55E+06	1.03E+06	0.39	0.34
11	2.90E+07	5.00E+06	0.76	1.90E+07	4.60E+06	0.62	2.55E+06	4.10E+05	0.79	0.72
12	2.90E+07	7.80E+06	0.57	1.90E+07	4.60E+06	0.62	2.55E+06	5.10E+05	0.70	0.63
13	6.00E+06	2.20E+06	0.44	2.70E+06	1.60E+06	0.23	1.65E+06	9.50E+05	0.24	0.30
14	6.00E+06	2.60E+06	0.36	2.70E+06	1.11E+06	0.39	1.65E+06	1.12E+06	0.17	0.31
15	6.00E+06	3.00E+05	1.30	2.70E+06	3.00E+04	1.95	1.65E+06	7.50E+04	1.34	1.53
16	6.00E+06	1.00E+05	1.78	2.70E+06	3.30E+04	1.91	1.65E+06	3.40E+04	1.69	1.79

Table A-2. $2^{5\text{-}1}$ Factional Factorial Design (Modified m-FC method)

Run	Replicate 1			Replicate 2			Replicate 3			Average Reduction
	Primary solution	After freezing	Reduction	Primary solution	After freezing	Reduction	Primary solution	After freezing	Reduction	
	(CFU/mL)	(CFU/mL)	$-\log N/N_0$	(CFU/mL)	(CFU/mL)	$-\log N/N_0$	(CFU/mL)	(CFU/mL)	$-\log N/N_0$	$-\log N/N_0$
1	3.30E+07	9.00E+06	0.56	1.95E+07	9.50E+06	0.31	3.00E+07	6.50E+06	0.66	0.51
2	3.30E+07	9.10E+06	0.56	1.95E+07	4.60E+06	0.63	3.00E+07	4.30E+06	0.84	0.68
3	3.30E+07	2.20E+06	1.18	1.95E+07	1.86E+06	1.02	3.00E+07	1.70E+06	1.25	1.15
4	3.30E+07	7.50E+06	0.64	1.95E+07	2.90E+06	0.83	3.00E+07	2.30E+06	1.12	0.86
5	9.80E+06	2.48E+06	0.60	1.39E+07	1.00E+06	1.14	1.20E+07	3.90E+06	0.49	0.74
6	9.80E+06	3.40E+06	0.46	1.39E+07	2.50E+06	0.75	1.20E+07	4.90E+06	0.39	0.53
7	9.80E+06	9.50E+05	1.01	1.39E+07	8.00E+04	2.24	1.20E+07	4.60E+05	1.42	1.56
8	9.80E+06	1.00E+05	1.99	1.39E+07	6.80E+04	2.31	1.20E+07	2.50E+05	1.68	1.99
9	2.90E+07	1.21E+07	0.38	1.90E+07	1.21E+07	0.20	2.55E+06	1.23E+06	0.32	0.30
10	2.90E+07	1.08E+07	0.43	1.90E+07	1.16E+07	0.21	2.55E+06	1.50E+06	0.23	0.29
11	2.90E+07	4.20E+06	0.84	1.90E+07	4.25E+06	0.65	2.55E+06	3.70E+05	0.84	0.78
12	2.90E+07	1.10E+07	0.42	1.90E+07	4.60E+06	0.62	2.55E+06	5.40E+05	0.67	0.57
13	6.00E+06	3.10E+06	0.29	2.70E+06	1.20E+06	0.35	1.65E+06	1.13E+06	0.16	0.27
14	6.00E+06	3.50E+06	0.23	2.70E+06	1.42E+06	0.28	1.65E+06	1.16E+06	0.15	0.22
15	6.00E+06	2.00E+05	1.48	2.70E+06	5.50E+04	1.69	1.65E+06	9.20E+04	1.25	1.47
16	6.00E+06	2.00E+05	1.48	2.70E+06	3.50E+04	1.89	1.65E+06	3.50E+04	1.67	1.68

1.2. *E.coli* reduction in different growth phase

Table A-3. *E.coli* reduction vs. growth time (m-FC method)

	Replicate 1.					
Time	Before freezing		After freezing		Reduction	
(hrs)	(CFU/mL)	Log N_0	(CFU/mL)	Log N	(%)	(-log N/N_0)
0	3.60E+06	6.56				
0.5	4.80E+06	6.68	8.40E+05	5.92	82.50	0.76
2	3.00E+06	6.48	4.00E+04	4.60	98.67	1.88
4	4.00E+07	7.60	1.00E+04	4.00	99.98	3.60
6	4.50E+08	8.65	1.00E+06	6.00	99.78	2.65
8	1.03E+09	9.01	1.46E+08	8.16	85.83	0.85
24	1.00E+09	9.00	1.30E+08	8.11	87.00	0.89
72	1.30E+09	9.11	7.00E+07	7.85	94.62	1.27
120	9.80E+08	8.99	6.00E+07	7.78	93.88	1.21
192	8.50E+08	8.93	1.00E+07	7.00	98.82	1.93
240	7.20E+08	8.86	5.40E+07	7.73	92.50	1.12
336	2.60E+08	8.41				
384	4.30E+07	7.63	1.00E+03	3.00	99.998	4.63
	Replicate 2.					
0	4.00E+06	6.60				
0.5	3.90E+06	6.59	8.80E+05	5.94	77.44	0.65
1	4.20E+06	6.62	4.50E+05	5.65	89.29	0.97
3	5.10E+06	6.71	3.60E+04	4.56	99.29	2.15
5	7.90E+07	7.90	2.00E+03	3.30	99.997	4.60
7	6.80E+08	8.83	2.55E+07	7.41	96.25	1.43
24	1.16E+09	9.06	2.00E+08	8.30	82.76	0.76
30	1.22E+09	9.09	3.50E+08	8.54	71.31	0.54
48	1.19E+09	9.08	3.75E+08	8.57	68.49	0.50
72	1.22E+09	9.09	1.09E+08	8.04	91.07	1.05
96	1.28E+09	9.11	1.60E+08	8.20	87.50	0.90
120	1.06E+09	9.03	1.60E+08	8.20	84.91	0.82
144	1.08E+09	9.03	6.50E+07	7.81	93.98	1.22
168	1.21E+09	9.08	3.20E+07	7.51	97.36	1.58
192	1.20E+09	9.08	2.30E+06	6.36	99.81	2.72
216	1.05E+09	9.02				
264	7.90E+08	8.90	7.00E+04	4.85	99.991	4.05
360	4.20E+08	8.62				
384	3.00E+07	7.48	7.00E+02	2.85	99.998	4.63

(Continued of Table A-3)

	Replicate 3.					
Time	Before freezing		After freezing		Reduction	
(hrs)	(CFU/mL)	Log N_0	(CFU/mL)	Log N	(%)	(-log N/N_0)
0	3.10E+06	6.49				
2	2.80E+06	6.45	2.20E+04	4.34	99.21	2.10
4	1.20E+07	7.08	7.00E+03	3.85	99.94	3.23
7	3.80E+08	8.58				
24	6.10E+08	8.79	6.00E+07	7.78	90.16	1.01
30	6.90E+08	8.84	6.00E+07	7.78	91.30	1.06
48	7.90E+08	8.90	8.00E+07	7.90	89.87	0.99
72	8.00E+08	8.90	1.40E+07	7.15	98.25	1.76
96	1.02E+09	9.01	6.00E+06	6.78	99.41	2.23
120	8.40E+08	8.92	5.40E+06	6.73	99.36	2.19
144	5.80E+08	8.76	2.30E+06	6.36	99.60	2.40
168	5.70E+08	8.76	6.00E+05	5.78	99.89	2.98
196	5.50E+08	8.74	4.00E+04	4.60	99.99	4.14
216	3.30E+08	8.52	1.40E+05	5.15	99.96	3.37
240	2.60E+08	8.41				
264	1.10E+08	8.04	1.90E+05	5.28	99.83	2.76
288	6.00E+07	7.78	1.60E+04	4.20	99.97	3.57
	Replicate 4.					
24	1.70E+09	9.23	8.40E+07	7.92	95.06	1.31
48	1.57E+09	9.20	4.60E+07	7.66	97.07	1.53
72	1.51E+09	9.18	4.90E+07	7.69	96.75	1.49
96	1.39E+09	9.14	5.90E+07	7.77	95.76	1.37
120	1.32E+09	9.12	6.60E+07	7.82	95.00	1.30
168	1.18E+09	9.07	5.80E+07	7.76	95.08	1.31
192	1.30E+09	9.11	1.80E+07	7.26	98.62	1.86
216	1.30E+09	9.11	2.90E+07	7.46	97.77	1.65
240	1.57E+09	9.20	2.40E+07	7.38	98.47	1.82
312	6.40E+08	8.81	9.60E+06	6.98	98.50	1.82

Table A-4. *E.coli* reduction vs. growth time (Modified m-FC method)

	Replicate 1.					
Time	Before freezing		After freezing		Reduction	
(hrs)	(CFU/mL)	Log N_0	(CFU/mL)	Log N	(%)	(-log N/N_0)
0	3.60E+06	6.56				
0.5	3.80E+06	6.58	8.40E+05	5.92	77.89	0.66
2	7.00E+06	6.85	5.00E+04	4.70	99.29	2.15
4	2.80E+07	7.45	1.00E+04	4.00	99.96	3.45
6	4.50E+08	8.65	1.00E+06	6.00	99.78	2.65
8	7.80E+08	8.89	2.00E+08	8.30	74.36	0.59
24	1.18E+09	9.07	1.60E+08	8.20	86.44	0.87
72	1.08E+09	9.03	1.50E+08	8.18	86.11	0.86
120	1.19E+09	9.08	6.00E+07	7.78	94.96	1.30
192	9.80E+08	8.99	1.00E+07	7.00	98.98	1.99
240	7.20E+08	8.86	3.20E+07	7.51	95.56	1.35
288	4.70E+08	8.67	5.00E+05	5.70	99.89	2.97
336	2.50E+08	8.40	1.00E+05	5.00	99.96	3.40
384	6.30E+07	7.80	5.00E+03	3.70	99.99	4.10
	Replicate 2.					
0	4.00E+06	6.60				
0.5	3.70E+06	6.57	8.80E+05	5.94	76.22	0.62
1	3.80E+06	6.58	4.50E+05	5.65	88.16	0.93
3	5.10E+06	6.71	3.60E+04	4.56	99.29	2.15
5	7.90E+07	7.90	2.00E+03	3.30	99.997	4.60
7	6.80E+08	8.83	2.55E+07	7.41	96.25	1.43
24	1.48E+09	9.17	2.00E+08	8.30	86.49	0.87
30	1.46E+09	9.16	3.50E+08	8.54	76.03	0.62
48	1.19E+09	9.08	3.75E+08	8.57	68.49	0.50
72	1.28E+09	9.11	1.09E+08	8.04	91.48	1.07
96	1.28E+09	9.11	1.60E+08	8.20	87.50	0.90
120	1.37E+09	9.14	1.60E+08	8.20	88.32	0.93
144	1.40E+09	9.15	6.50E+07	7.81	95.36	1.33
168	1.38E+09	9.14	5.70E+07	7.76	95.87	1.38
192	1.09E+09	9.04	7.00E+06	6.85	99.36	2.19
216	1.01E+09	9.00	6.00E+05	5.78	99.94	3.23
264	7.80E+08	8.89	7.00E+05	5.85	99.91	3.05
360	5.80E+07	7.76	1.20E+05	5.08	99.79	2.68
384	1.70E+07	7.23	9.00E+02	2.95	99.99	4.28

(Continued of Table A-4)

	Replicate 3.					
Time	Before freezing		After freezing		Reduction	
(hrs)	(CFU/mL)	Log N_0	(CFU/mL)	Log N	(%)	(-log N/N_0)
0	3.50E+06	6.54				
2	2.90E+06	6.46	3.20E+04	4.51	98.90	1.96
4	1.41E+07	7.15	6.50E+03	3.81	99.95	3.34
7	4.10E+08	8.61	1.06E+07	7.03	97.41	1.59
10	5.00E+08	8.70	0.00E+00			
24	9.40E+08	8.97	1.03E+08	8.01	89.04	0.96
30	9.30E+08	8.97	2.10E+08	8.32	77.42	0.65
33	7.80E+08	8.89	0.00E+00			
48	7.80E+08	8.89	1.30E+08	8.11	83.33	0.78
54	7.70E+08	8.89	1.08E+08	8.03	85.97	0.85
72	9.60E+08	8.98	4.00E+07	7.60	95.83	1.38
96	8.80E+08	8.94	2.00E+07	7.30	97.73	1.64
120	8.50E+08	8.93	1.50E+07	7.18	98.24	1.75
144	7.80E+08	8.89	6.50E+06	6.81	99.17	2.08
168	6.20E+08	8.79	2.50E+06	6.40	99.60	2.39
196	6.50E+08	8.81	3.70E+05	5.57	99.94	3.24
216	5.20E+08	8.72	4.80E+05	5.68	99.91	3.03
240	4.50E+08	8.65	1.20E+06	6.08	99.73	2.57
264	1.20E+08	8.08	1.90E+05	5.28	99.84	2.80
288	5.50E+07	7.74	1.40E+04	4.15	99.97	3.59
	Replicate 4.					
24	1.36E+09	9.13	2.70E+08	8.43	80.15	0.70
48	1.31E+09	9.12	9.00E+07	7.95	93.13	1.16
72	1.37E+09	9.14	1.18E+08	8.07	91.39	1.06
96	1.49E+09	9.17	1.21E+08	8.08	91.88	1.09
120	1.45E+09	9.16	9.10E+07	7.96	93.72	1.20
168	1.10E+09	9.04	5.50E+07	7.74	95.00	1.30
192	1.09E+09	9.04	2.30E+07	7.36	97.89	1.68
216	1.50E+09	9.18	3.40E+07	7.53	97.73	1.64
240	1.80E+09	9.26	3.50E+07	7.54	98.06	1.71
312	5.60E+08	8.75	1.50E+07	7.18	97.32	1.57

1.3. Freezing storage time vs. *E.coli* reduction

1.3.1. m-FC method

Table A-5. *E.coli* removal vs. freezing storage time (Freezing & storage at -35°C)

	Rep.1	Reduction		Rep.2	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	3.50E+06			5.00E+06		
Frozed w/o storage	2.90E+05	93.41	1.181	4.20E+05	91.60	1.076
Storage 2 days	2.00E+05	95.45	1.342	4.30E+05	91.40	1.066
Storage 5 days	2.30E+05	94.77	1.282	2.80E+05	94.40	1.252
Storage 10 days	2.40E+05	94.55	1.263	3.10E+05	93.80	1.208
Storage 20 days	2.60E+05	94.09	1.228	3.90E+05	92.20	1.108
	Rep.3	Reduction		Rep.4	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	4.30E+06			7.70E+06		
Frozed w/o storage	2.30E+05	94.18	1.235	8.00E+05	89.61	0.983
Storage 2 days	3.60E+05	90.89	1.040	3.10E+05	95.97	1.395
Storage 5 days	2.00E+05	94.94	1.296	2.80E+05	96.36	1.439
Storage 10 days	1.70E+05	95.70	1.366	1.90E+05	97.53	1.608
Storage 20 days	3.70E+05	90.63	1.028	1.90E+05	97.53	1.608
Storage 30 days	8.00E+04	97.97	1.694	1.70E+05	97.79	1.656
	Rep.5	Reduction		Rep.6	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	1.12E+07			9.30E+06		
Frozed w/o storage	6.00E+05	94.64	1.271	3.00E+05	96.77	1.491
Storage 2 days	7.40E+05	93.39	1.180			
Storage 5 days	1.07E+06	90.45	1.020			
Storage 10 days	9.40E+05	91.61	1.076	4.50E+05	95.16	1.315
Storage 20 days	3.70E+05	96.70	1.481	5.00E+05	94.62	1.270
Storage 30 days	5.30E+05	95.27	1.325	2.70E+05	97.10	1.537

Table A-6. *E.coli* reduction vs. freezing storage time (Freezing & storage at -15°C)

	Rep.1	Reduction		Rep.2	Reduction 1	
	(CFU/mL)	(%)	$-\log (N/N_0)$	(CFU/mL)	(%)	$-\log (N/N_0)$
Original Number	3.50E+06			5.00E+06		
Frozen w/o storage	1.10E+06	75.00	0.602	1.30E+06	74.00	0.585
Storage 2 days	4.10E+05	90.68	1.031	5.30E+05	89.40	0.975
Storage 5 days	6.50E+05	85.23	0.831			
Storage 10 days	2.90E+05	93.41	1.181	3.60E+05	92.80	1.143
Storage 20 days	2.00E+05	95.45	1.342	3.10E+05	93.80	1.208
Storage 30 days	1.90E+05	95.68	1.365	4.50E+05	91.00	1.046
	Rep.3	Reduction		Rep.4	Reduction	
	(CFU/mL)	(%)	$-\log (N/N_0)$	(CFU/mL)	(%)	$-\log (N/N_0)$
Original Number	3.95E+06			7.70E+06		
Frozen w/o storage	8.00E+05	79.75	0.694	1.00E+06	87.01	0.886
Storage 2 days	5.80E+05	85.32	0.833	6.50E+05	91.56	1.074
Storage 5 days	3.80E+05	90.38	1.017	5.20E+05	93.25	1.170
Storage 10 days	1.40E+05	96.46	1.450	3.70E+05	95.19	1.318
Storage 20 days	1.50E+05	96.20	1.421	9.00E+04	98.83	1.932
Storage 30 days	5.00E+04	98.73	1.898	7.00E+04	99.09	2.041
	Rep.5	Reduction		Rep.6	Reduction	
	(CFU/mL)	(%)	$-\log (N/N_0)$	(CFU/mL)	(%)	$-\log (N/N_0)$
Original Number	3.55E+06			3.55E+06		
Frozen w/o storage	5.00E+05	85.92	0.851	1.60E+06	59.49	0.392
Storage 2 days	4.40E+05	87.61	0.907	2.00E+05	94.94	1.296
Storage 5 days	2.70E+05	92.39	1.119	1.70E+05	95.70	1.366
Storage 10 days	2.60E+05	92.68	1.135	7.00E+04	98.23	1.751
Storage 20 days	8.00E+04	97.75	1.647	2.00E+04	99.49	2.296
Storage 30 days	8.00E+04	97.75	1.647	1.50E+05	96.20	1.421

Table A-7. *E.coli* reduction vs. freezing storage time (Freezing & storage at -5°C)

	Rep.1	Reduction		Rep.2	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	4.65E+06			2.14E+07		
Frozed w/o storage	5.00E+05	89.25	0.968	2.45E+06	88.55	0.941
Storage 2 days	2.80E+05	93.98	1.220	7.10E+05	96.68	1.479
Storage 5 days				8.60E+05	95.98	1.396
Storage 10 days	1.64E+05	96.47	1.453	4.50E+05	97.90	1.677
Storage 20 days	5.33E+04	98.85	1.940	3.00E+05	98.60	1.853
Storage 30 days	3.40E+04	99.27	2.136	1.40E+05	99.35	2.184
	Rep.3	Reduction		Rep.4	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	3.85E+06			3.75E+06		
Frozed w/o storage	3.50E+05	92.47	1.123	3.50E+05	98.36	1.786
Storage 2 days	6.00E+04	98.71	1.889			
Storage 5 days	2.80E+04	99.40	2.220	2.15E+04	99.90	2.998
Storage 10 days	5.33E+03	99.89	2.940	9.00E+03	99.96	3.376
Storage 20 days	9.50E+03	99.80	2.690	1.04E+04	99.95	3.313
Storage 30 days	1.38E+04	99.70	2.528	8.20E+03	99.96	3.417

1.3.2. Modified m-FC method

Table A-8. *E.coli* reduction vs. freezing storage time (Freezing & storage at -35°C)

	Rep.1	Reduction		Rep.2	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	4.40E+06			4.35E+06		
Frozen w/o storage	1.08E+06	75.45	0.61	1.06E+06	75.63	0.61
Storage 2 days	7.60E+05	82.73	0.76	7.20E+05	83.45	0.78
Storage 5 days	7.90E+05	82.05	0.75	8.00E+05	81.61	0.74
Storage 10 days	6.60E+05	85.00	0.82	6.20E+05	85.75	0.85
Storage 20 days	5.10E+05	88.41	0.94	7.20E+05	83.45	0.78
Storage 30 days				6.80E+05	84.37	0.81
	Rep.3	Reduction		Rep.4	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	3.95E+06			6.40E+06		
Frozen w/o storage	1.14E+06	71.14	0.54	1.41E+06	77.97	0.66
Storage 2 days	9.40E+05	76.20	0.62	7.60E+05	88.13	0.93
Storage 5 days	5.30E+05	86.58	0.87	6.80E+05	89.38	0.97
Storage 10 days	4.40E+05	88.86	0.95	5.50E+05	91.41	1.07
Storage 20 days	5.10E+05	87.09	0.89	3.70E+05	94.22	1.24
Storage 30 days	2.55E+05	93.54	1.19	3.90E+05	93.91	1.22
	Rep.5	Reduction		Rep.6	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	1.06E+07			9.20E+06		
Frozen w/o storage	3.00E+06	71.70	0.55	1.90E+06	79.35	0.69
Storage 2 days	1.63E+06	84.62	0.81			
Storage 5 days	1.92E+06	81.89	0.74			
Storage 10 days	1.58E+06	85.09	0.83	1.04E+06	88.70	0.95
Storage 20 days	9.10E+05	91.42	1.07	8.50E+05	90.76	1.03
Storage 30 days	5.20E+05	95.09	1.31	7.20E+05	90.76	1.03

Table A-9. *E.coli* reduction vs. freezing storage time (Freezing & storage at -15°C)

	Rep.1	Reduction		Rep.2	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	4.40E+06			4.35E+06		
Frozed w/o storage	2.20E+06	50.00	0.30	2.00E+06	54.02	0.34
Storage 2 days	1.40E+06	68.18	0.50	1.18E+06	72.87	0.57
Storage 5 days	1.30E+06	70.45	0.53	2.10E+06	51.72	0.32
Storage 10 days	1.25E+06	71.59	0.55	9.60E+05	77.93	0.66
Storage 15 days	1.05E+06	76.14	0.62			
Storage 20 days				3.00E+05	93.10	1.16
Storage 30 days						
	Rep.3	Reduction		Rep.4	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	3.95E+06			6.40E+06		
Frozed w/o storage	2.20E+06	44.30	0.25	1.98E+06	69.06	0.51
Storage 2 days	8.10E+05	79.49	0.69	1.40E+06	78.13	0.66
Storage 5 days	6.30E+05	84.05	0.80	9.10E+05	85.78	0.85
Storage 10 days	3.30E+05	91.65	1.08	8.20E+05	87.19	0.89
Storage 15 days				2.25E+05	96.48	1.45
Storage 20 days				1.70E+05	97.34	1.58
Storage 30 days	1.15E+05	97.09	1.54	1.60E+05	97.50	1.60
	Rep.5	Reduction		Rep.6	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	3.85E+06			3.85E+06		
Frozed w/o storage	1.58E+06	58.96	0.39	1.90E+06	50.65	0.31
Storage 2 days	6.40E+05	83.38	0.78	5.50E+05	85.71	0.85
Storage 5 days	6.70E+05	82.60	0.76	3.90E+05	89.87	0.99
Storage 10 days	3.00E+05	92.21	1.11	2.90E+05	92.47	1.12
Storage 15 days	4.40E+05	88.57	0.94	3.40E+05	91.17	1.05
Storage 20 days	1.30E+05	96.62	1.47	1.80E+05	95.32	1.33
Storage 30 days	1.30E+05	96.62	1.47	2.30E+05	94.03	1.22

Table A-10. *E.coli* reduction vs. freezing storage time (Freezing & storage at -5°C)

	Rep.1	Reduction		Rep.2	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	4.65E+06			2.14E+07		
Frozed w/o storage	6.50E+05	86.02	0.855	3.00E+06	85.98	0.853
Storage 2 days	2.80E+05	93.98	1.220	6.80E+05	96.82	1.498
Storage 5 days	1.60E+05	96.56	1.463	9.20E+05	95.70	1.367
Storage 10 days	1.76E+05	96.22	1.422	4.80E+05	97.76	1.649
Storage 20 days	3.67E+04	99.21	2.103	2.70E+05	98.74	1.899
Storage 30 days	2.00E+04	99.57	2.366	1.47E+05	99.31	2.164
	Rep.3	Reduction		Rep.4	Reduction	
	(CFU/mL)	(%)	-log (N/N_0)	(CFU/mL)	(%)	-log (N/N_0)
Original Number	3.85E+06			3.75E+06		
Frozed w/o storage	3.50E+05	90.91	1.041	4.30E+05	88.53	0.941
Storage 2 days	4.00E+04	98.96	1.983	7.00E+04	98.13	1.729
Storage 5 days	2.20E+04	99.43	2.243	3.10E+04	99.17	2.083
Storage 10 days	1.07E+04	99.72	2.557	1.30E+04	99.65	2.460
Storage 20 days	1.16E+04	99.70	2.521	1.21E+04	99.68	2.491
Storage 30 days	1.82E+04	99.53	2.325	9.80E+03	99.74	2.583

1.4. Effect of freezing cycle to *E.coli* removal

1.4.1. m-FC method

Table A-11. Freezing cycle vs. *E.coli* reduction (Freezing at -35°C)

	Rep 1.			Rep 2.		
Freezing	Number	Reduction		Number	Reduction	
cycle	(CFU/mL)	(%)	(-log N/N_0)	(CFU/mL)	(%)	(-log N/N_0)
Original Number	4.05E+06			4.30E+06		
1	3.55E+05	91.235	1.06	6.20E+05	85.581	0.84
2	1.08E+05	97.333	1.57	2.10E+05	95.116	1.31
3	2.20E+04	99.457	2.27			
4				8.00E+03	99.814	2.73
5	1.40E+03	99.965	3.46			
6				1.00E+02	99.998	4.63

	Rep 3.		
Freezing	Number	Reduction	
cycle	(CFU/mL)	(%)	(-log N/N_0)
Original Number	9.30E+06		
1	3.00E+05	96.774	1.49
2	1.60E+05	98.280	1.76
3	1.48E+05	98.409	1.80
4	6.50E+04	99.301	2.16
5	1.50E+04	99.839	2.79
6	7.90E+03	99.915	3.07

Table A-12. Freezing cycle vs. *E.coli* reduction (Freezing at -15°C)

	Rep 1.			Rep 2.		
Freezing cycle	Number (CFU/mL)	Reduction		Number (CFU/mL)	Reduction	
		(%)	$(-\log N/N_0)$		(%)	$(-\log N/N_0)$
Original Number	4.05E+06			4.30E+06		
1	1.16E+06	71.358	0.54	8.60E+05	80.00	0.70
2	2.30E+05	94.321	1.25	3.20E+05	92.56	1.13
3	4.30E+04	98.938	1.97			
4				1.30E+04	99.70	2.52
5	5.00E+03	99.877	2.91			
	Rep 3.			Rep 4.		
Original Number	3.55E+06			9.30E+06		
1	6.00E+05	83.099	0.77	1.70E+06	81.720	0.74
2	3.00E+05	91.549	1.07	8.30E+05	91.075	1.05
3	1.63E+05	95.408	1.34	6.80E+05	92.688	1.14
4	5.70E+04	98.394	1.79	5.80E+05	93.763	1.21
5	9.00E+03	99.746	2.60			

Table A-13. Freezing cycle vs. *E.coli* reduction (Freezing at -5°C)

	Rep 1.			Rep 2.		
Freezing cycle	Number (CFU/mL)	Reduction		Number (CFU/mL)	Reduction	
		(%)	$(-\log N/N_0)$		(%)	$(-\log N/N_0)$
Original Number	4.65E+06			2.14E+07		
1	5.00E+05	89.247	0.97	2.45E+06	88.55	0.94
2				3.00E+05	98.60	1.85
3	2.27E+04	99.513	2.31	2.60E+05	98.79	1.92
4	9.40E+03	99.798	2.69	8.29E+04	99.61	2.41
5	1.80E+03	99.961	3.41	2.40E+04	99.89	2.95
6	3.10E+02	99.993	4.18			
	Rep 3.			Rep 4.		
Original Number	3.85E+06			3.75E+06		
1	5.00E+05	87.013	0.89	3.50E+05	90.667	1.03
2	1.40E+04	99.636	2.44	5.10E+04	98.640	1.87
3	6.30E+03	99.836	2.79	1.90E+04	99.493	2.30
4	2.90E+03	99.925	3.12	6.90E+03	99.816	2.74
5	1.10E+03	99.971	3.54	2.27E+03	99.940	3.22
6	5.00E+01	99.999	4.89	4.71E+02	99.987	3.90

1.4.2. Modified m-FC method

Table A-14. Freezing cycle vs. *E.coli* reduction (Freezing at -35°C)

	Rep 1.			Rep 2.		
Freezing	Number	Reduction		Number	Reduction	
cycle	(CFU/mL)	(%)	(-log N/N_0)	(CFU/mL)	(%)	(-log N/N_0)
Original Number	4.05E+06			4.20E+06		
1	1.26E+06	68.89	0.507	1.14E+06	72.857	0.566
2	1.70E+05	95.80	1.377	4.40E+05	89.524	0.980
3	3.90E+04	99.04	2.016			
4				1.68E+04	99.600	2.398
5	1.70E+03	99.96	3.377			
6				8.20E+02	99.980	3.709
7	1.20E+02	100.00	4.528	3.45E+02	99.992	4.085

	Rep 3.		
Freezing	Number	Reduction	
cycle	(CFU/mL)	(%)	(-log N/N_0)
Original Number	9.60E+06	(%)	(-log N/N_0)
1	1.90E+06	80.208	0.704
2	3.80E+05	96.042	1.402
3	1.30E+05	98.646	1.868
4	1.03E+05	98.927	1.969
5	2.50E+04	99.740	2.584
6	7.50E+03	99.922	3.107

Table A-15. Freezing cycle vs. *E.coli* reduction (Frozen at -15°C)

	Rep 1.			Rep 2.		
Freezing cycle	Number (CFU/mL)	Reduction (%)	(-log N/N_0)	Number (CFU/mL)	Reduction (%)	(-log N/N_0)
Original Number	4.05E+06			4.20E+06		
1	2.10E+06	48.148	0.29	1.90E+06	54.762	0.34
2	4.60E+05	88.642	0.94	6.00E+05	85.714	0.85
3	8.65E+04	97.864	1.67			
4				2.00E+05	95.238	1.32
5	5.00E+03	99.877	2.91			
	Rep 3.			Rep 4.		
Original Number	3.85E+06			9.60E+06		
1	1.70E+06	55.844	0.36	2.90E+06	69.792	0.52
2	4.60E+05	88.052	0.92	1.50E+06	84.375	0.81
3	2.40E+05	93.766	1.21	1.23E+06	87.240	0.89
4	7.60E+04	98.026	1.70	7.30E+05	92.396	1.12
5	2.20E+04	99.429	2.24	5.00E+05	94.792	1.28
6	2.10E+04	99.455	2.26	3.70E+05	96.146	1.41

Table A-16. Freezing cycle vs. *E.coli* reduction (Frozen at -5°C)

	Rep. 1.			Rep. 2.		
Freezing	Number	Reduction		Number	Reduction	
cycle	(CFU/mL)	(%)	(-log N/N_0)	(CFU/mL)	(%)	(-log N/N_0)
Original Number	4.65E+06			2.14E+07		
1	6.50E+05	86.022	0.85	3.00E+06	85.981	0.85
2				3.40E+05	98.411	1.80
3	2.40E+04	99.484	2.29	3.83E+05	98.209	1.75
4	1.05E+04	99.774	2.65	9.14E+04	99.573	2.37
5	2.00E+03	99.957	3.37	2.50E+04	99.883	2.93
6	2.70E+02	99.994	4.24			
	Rep. 3.			Rep. 4.		
Original Number	3.85E+06			3.75E+06		
1	5.00E+05	87.013	0.89	4.30E+05	88.53	0.94
2	1.05E+04	99.727	2.56	3.40E+04	99.09	2.04
3	8.40E+03	99.782	2.66	2.60E+04	99.31	2.16
4	3.10E+03	99.919	3.09	8.00E+03	99.79	2.67
5	1.00E+02	99.997	4.59	3.00E+03	99.92	3.10
6	7.00E+01	99.998	4.74	5.86E+02	99.98	3.81

1.5. Spraying freezing test for *E.coli* reduction

Table A-17. Spraying freezing test for *E.coli* reduction (Flow rate: 20.0 mL/min.)

Rep.1	Mm-FC	Reduction	Reduction	m-FC	Reduction	Reduction
	(CFU/mL)	(%)	(-logN/N_0)	(CFU/mL)	(%)	(-logN/N_0)
Original	1.50E+06			1.50E+06		
Run off	2.30E+06	-53.33	-0.19	2.00E+06	-33.33	-0.125
Melted ice	7.40E+05	50.67	0.31	9.30E+05	38.00	0.208
Rep.2	m-FC	Reduction	Reduction	Mm-FC	Reduction	Reduction
	(CFU/mL)	(%)	(-logN/N_0)	(CFU/mL)	(%)	(-logN/N_0)
Original	2.30E+06			2.30E+06		
Run off	3.70E+06	-60.87	-0.21	2.50E+06	-8.70	-0.036
Melted ice	1.06E+06	53.91	0.34	9.50E+05	58.70	0.384
Rep.3	m-FC	Reduction	Reduction	Mm-FC	Reduction	Reduction
	(CFU/mL)	(%)	(-logN/N_0)	(CFU/mL)	(%)	(-logN/N_0)
Original	3.20E+06			3.20E+06		
Run off	4.70E+06	-46.88	-0.17	4.00E+06	-25.00	-0.097
Melted ice	1.60E+06	50.00	0.30	1.55E+06	51.56	0.315
Rep.4	m-FC	Reduction	Reduction	Mm-FC	Reduction	Reduction
	(CFU/mL)	(%)	(-logN/N_0)	(CFU/mL)	(%)	(-logN/N_0)
Original	3.10E+06			3.10E+06		
Run off	4.60E+06	-48.39	-0.17	3.80E+06	-22.58	-0.088
Melted ice	1.38E+06	55.48	0.52	1.30E+06	58.06	0.377

Table A-18. Spraying test for *E.coli* reduction without storage (Flow rate: 24.1 ml/min.)

Rep.1	Volume	Melted	Accumulate	m-FC	Reduction	Mm-FC	Reduction
	(mL)	Percent (%)		(CFU/mL)	(-log N/N_0)	(CFU/mL)	(-log N/N_0)
Original				2.10E+06		2.10E+06	
Run off				1.00E+06	0.322	7.33E+05	0.457
Melted ice	47.5	20.79	20.79	8.00E+05	0.419	1.00E+06	0.322
	55.0	24.07	44.86	1.75E+06	0.079	1.40E+06	0.176
	59.0	25.82	70.68	8.40E+05	0.398	7.40E+05	0.453
	67.0	29.32	100.00	6.40E+05	0.516	6.90E+05	0.483
Total	228.5	100.00		9.92E+05	0.326	9.38E+05	0.350
Rep.2	Volume	Melted	Accumulate	m-FC	Reduction	Mm-FC	Reduction
	(mL)	Percent (%)		(CFU/mL)	(-log N/N_0)	(CFU/mL)	(-log N/N_0)
Original				2.05E+06		2.05E+06	
Melted ice	68.5	28.90	28.90	1.40E+06	0.166	1.25E+06	0.215
	49.0	20.68	49.58	5.00E+05	0.613	5.00E+05	0.613
	44.5	18.78	68.35	3.20E+05	0.807	3.80E+05	0.732
	75.0	31.65	100.00	1.20E+05	1.233	1.50E+05	1.136
Total	237.0	100.00		6.06E+05	0.529	5.83E+05	0.546
Rep.3	Volume	Melted	Accumulate	m-FC	Reduction	Mm-FC	Reduction
	(mL)	Percent (%)		(CFU/mL)	(-log N/N_0)	(CFU/mL)	(-log N/N_0)
Original				1.85E+06		1.85E+06	
Run off				2.00E+06	-0.034	2.33E+06	-0.101
Melted ice	19.0	8.53	8.53	1.80E+06	0.012	2.07E+06	-0.048
	57.0	25.58	34.11	1.72E+06	0.032	1.36E+06	0.134
	64.8	29.08	63.20	6.70E+05	0.441	8.20E+05	0.353
	82.0	36.80	100.00	6.50E+05	0.454	7.00E+05	0.422
Total	222.8	100.00		1.03E+06	0.255	1.02E+06	0.258
Rep.4	Volume	Melted	Accumulate	m-FC	Reduction	Mm-FC	Reduction
	(mL)	Percent (%)		(CFU/mL)	(-log N/N_0)	(CFU/mL)	(-log N/N_0)
Original				1.80E+06		1.80E+06	
Run off				1.73E+06	0.016	2.07E+06	-0.060
Melted ice	20.6	11.31	11.31	2.40E+06	-0.125	2.00E+06	-0.046
	44.5	24.44	35.75	1.70E+06	0.025	1.30E+06	0.141
	59.0	32.40	68.15	6.50E+05	0.442	6.80E+05	0.423
	58.0	31.85	100.00	3.80E+05	0.675	3.90E+05	0.664
Total	182.1	100.00		1.02E+06	0.247	8.88E+05	0.307

Table A-19. Spraying test for *E.coli* removal with storage 2 days.

Rep.1	Volume	Melted	Accumulate	m-FC	Reduction	Mm-FC	Reduction
	(mL)	Percent (%)		(CFU/mL)	(-log N/N_0)	(CFU/mL)	(-log N/N_0)
Original				2.10E+06		2.10E+06	
Melted ice	27.2	20.12	20.12	5.00E+03	2.623	3.00E+03	2.845
	29.5	21.82	41.94	2.80E+03	2.875	3.50E+03	2.778
	31.0	22.93	64.87				
	47.5	35.13	100.00	8.10E+02	3.414	7.00E+02	3.477
Total	135.2	100.00					
Rep.2	Volume	Melted	Accumulate	m-FC	Reduction	Mm-FC	Reduction
	(mL)	Percent (%)		(CFU/mL)	(-log N/N_0)	(CFU/mL)	(-log N/N_0)
Original				2.05E+06		2.05E+06	
Melted ice	21.3	8.03	8.03	1.00E+02	4.312	2.00E+02	4.011
	63.5	23.94	31.96	2.00E+01	5.011	1.00E+02	4.312
	103.5	39.01	70.98	2.67E+01	4.885	2.00E+01	5.011
	77.0	29.02	100.00	1.00E+01	5.312	6.00E+00	5.534
Total	265.3	100.00		2.61E+01	4.895	4.95E+01	4.617
Rep.3	Volume	Melted	Accumulate	m-FC	Reduction	Mm-FC	Reduction
	(mL)	Percent (%)		(CFU/mL)	(-log N/N_0)	(CFU/mL)	(-log N/N_0)
Original				1.80E+06		1.80E+06	
Melted ice	23.9	21.19	21.19	4.33E+01	4.618	8.00E+01	4.352
	47.9	42.46	63.65	1.40E+01	5.109	1.90E+01	4.977
	41.0	36.35	100.00	1.82E+00	5.996	3.09E+00	5.765
Total	112.8	100.00		1.58E+01	5.057	2.61E+01	4.838
Rep.4	Volume	Melted	Accumulate	m-FC	Reduction	Mm-FC	Reduction
	(mL)	Percent (%)		(CFU/mL)	(-log N/N_0)	(CFU/mL)	(-log N/N_0)
Original				1.80E+06		1.80E+06	
Melted ice	30.5	37.47	37.47	1.68E+02	4.030	2.00E+02	3.954
	37.5	46.07	83.54	4.33E+01	4.618	7.17E+01	4.400
	13.4	16.46	100.00	3.33E+00	5.732	2.67E+00	5.829
Total	81.4	100.00		8.35E+01	4.334	1.08E+02	4.220

2. *Bacillus megaterium* Tests

2.1. The Effect of Freezing/Thawing to *Bacillus megaterium*

Table A-20. *Bacillus megaterium* reduction after freezing

Run		Dup. 1	Dup. 2	Ave.	Reduction -log N/N_0
Rep.1	Pre. Freezing	8.00E+05		8.00E+05	
	Frozen	7.10E+05	7.70E+05	7.40E+05	0.034
Rep.2	Pre. Freezing	9.00E+05		9.00E+05	
	Frozen	8.10E+05	8.80E+05	8.45E+05	0.027
Rep.3	Pre. Freezing	2.40E+05	2.70E+05	2.55E+05	
	Frozen	3.70E+05	1.90E+05	2.80E+05	-0.041
Rep.4	Pre. Freezing	4.50E+05	3.70E+05	4.10E+05	
	Frozen	4.10E+05	3.90E+05	4.00E+05	0.011
Rep.5	Pre. Freezing	3.50E+05	4.50E+05	4.00E+05	
	Frozen	3.90E+05		3.90E+05	0.011
Rep.6	Pre. Freezing	2.90E+05	3.00E+05	2.95E+05	
	Frozen	2.60E+05	3.00E+05	2.80E+05	0.023
Rep.7	Pre. Freezing	9.60E+05	9.50E+05	9.55E+05	
	Frozen	9.70E+05	1.05E+06	1.01E+06	-0.024
Rep.8	Pre. Freezing	9.70E+05	1.05E+06	1.01E+06	
	Frozen	9.70E+05	1.10E+06	1.04E+06	-0.011
Rep.9	Pre. Freezing	2.70E+05	2.40E+05	2.55E+05	
	Frozen	2.70E+05	2.10E+05	2.40E+05	0.026
Rep.10	Pre. Freezing	3.90E+05	3.50E+05	3.70E+05	
	Frozen	3.40E+05	4.10E+05	3.75E+05	-0.006

Note: Dup. = Duplicate

2.2. Spraying Freezing Test for *Bacillus megaterium* Reduction

Table A-21. Spraying freezing test for *Bacillus megaterium* reduction

	Without Storage				Storage for 2 days			
	Dup.1	Dup.2	Average	Reduction	Dup.1	Dup.2	Average	Reduction
Rep.1	(CFU/mL)			-log N/N_0	(CFU/mL)			-log N/N_0
Original	2.60E+05	2.80E+05	2.70E+05		2.60E+05	2.80E+05	2.70E+05	
Run off	2.50E+05	3.00E+05	2.75E+05	-0.008				
Melted	1.86E+05	3.14E+05	2.50E+05	0.033	2.65E+05		2.67E+05	0.005
Rep.2								
Original	2.60E+05	2.40E+05	2.50E+05		3.80E+05	4.70E+05	4.25E+05	
Run off	1.70E+05	1.00E+05	1.35E+05	0.268				
Melted		3.09E+05	3.09E+05	-0.092	3.83E+05	3.95E+05	3.89E+05	0.038
Rep.3								
Original	7.80E+05	7.90E+05	7.85E+05		7.80E+05	7.90E+05	7.85E+05	
Run off	5.40E+05	5.00E+05	5.20E+05	0.179				
Melted	7.00E+05	6.56E+05	6.78E+05	0.064	6.59E+05	6.58E+05	6.58E+05	0.076
Rep.4								
Original	5.40E+05	3.80E+05	4.60E+05		7.80E+05	7.90E+05	7.85E+05	
Run off	3.90E+05	5.00E+05	4.45E+05	0.010				
Melted	4.07E+05	3.77E+05	3.92E+05	0.064	8.16E+05	7.65E+05	7.90E+05	-0.003
Rep.5								
Original						3.80E+05	3.80E+05	
Melted					4.07E+05	3.77E+05	3.92E+05	-0.014

Note: Dup. = Duplicate; Rep.= Replicate

2.3. Chlorination for *Bacillus megaterium* Reduction

Table A-22. *Bacillus megaterium* reduction at 2.0 mg Cl_2/L.

Reduction without freezing					Reduction after freezing				
Rep.1	Dup. 1	Dup. 2	Average	Reduction -log N/N_0		Dup. 1	Dup. 2	Average	Reduction -log N/N_0
Working solution	1.37E+06	1.29E+06	1.33E+06		Before Freezing	8.00E+05		8.00E+05	
Original	1.15E+06	1.08E+06	1.12E+06		Frozen	7.10E+05	7.70E+05	7.40E+05	0.034
5 min	6.50E+05	5.70E+05	6.10E+05	0.263	Original	5.56E+05	6.03E+05	5.79E+05	
10 min	4.50E+05	4.20E+05	4.35E+05	0.410	5 min	4.30E+05	5.50E+05	4.90E+05	0.073
20 min	5.20E+05	3.80E+05	4.50E+05	0.395	10 min	3.20E+05	3.40E+05	3.30E+05	0.244
					20 min	1.90E+05	3.20E+05	2.55E+05	0.356
Rep.2					40 min	2.90E+05	3.00E+05	2.95E+05	0.293
Working solution	1.30E+06	1.40E+06	1.35E+06		Before Freezing	9.00E+05		9.00E+05	
Original	1.09E+06	1.18E+06	1.13E+06		Frozen	8.10E+05	8.80E+05	8.45E+05	0.027
5 min	7.40E+05	8.50E+05	7.95E+05	0.154	Original	6.37E+05	6.92E+05	6.64E+05	
10 min	4.80E+05	5.50E+05	5.15E+05	0.343	10 min	5.20E+05		5.20E+05	0.106
20 min	4.90E+05	5.10E+05	5.00E+05	0.356	20 min	4.10E+05	4.30E+05	4.20E+05	0.199
40 min	2.50E+05	2.60E+05	2.55E+05	0.648	40 min	3.60E+05	3.90E+05	3.75E+05	0.248
80 min.	2.70E+05	3.10E+05	2.90E+05	0.592					
Rep.3									
Working solution	7.20E+05	6.90E+05	7.05E+05		Before Freezing	2.40E+05	2.70E+05	2.55E+05	
Original	5.42E+05	5.20E+05	5.31E+05		Frozen	3.70E+05	1.90E+05	2.80E+05	-0.041
10 min	3.30E+05	3.20E+05	3.25E+05	0.213	Original	3.02E+05	1.55E+05	2.29E+05	

(Continued of Table A-22)

Reduction without freezing					Reduction after freezing				
Rep.3	Dup. 1	Dup. 2	Average	Reduction -log N/N$_0$		Dup. 1	Dup. 2	Average	Reduction -log N/N$_0$
20 min	2.60E+05	2.50E+05	2.55E+05	0.318	10 min	1.80E+05	1.50E+05	1.65E+05	0.142
40 min	1.90E+05	1.00E+05	1.45E+05	0.564	20 min	1.12E+05	1.01E+05	1.07E+05	0.332
Rep.4					40 min	6.90E+04	7.60E+04	7.25E+04	0.499
Working solution	4.20E+05	4.70E+05	4.45E+05		Before Freezing				
Original	3.41E+05	3.82E+05	3.62E+05		Frozen	3.50E+05	3.60E+05	3.55E+05	
10 min	2.10E+05	2.00E+05	2.05E+05	0.247	Original	3.18E+05	3.27E+05	3.22E+05	
20 min	1.80E+05	2.40E+05	2.10E+05	0.236	10 min	1.20E+05	1.50E+05	1.35E+05	0.378
40 min	2.20E+05	2.20E+05	2.20E+05	0.216	20 min	1.30E+05	1.10E+05	1.20E+05	0.429
					40 min	1.40E+05	1.10E+05	1.25E+05	0.411
Rep.5									
Working solution:	3.60E+05	4.50E+05	4.05E+05		Before Freezing	4.50E+05	3.70E+05	4.10E+05	
Original	3.53E+05	4.41E+05	3.97E+05		Frozen	4.10E+05	3.90E+05	4.00E+05	0.011
5 min	2.90E+05	2.10E+05	2.50E+05	0.201	Original	4.02E+05	3.82E+05	3.92E+05	
10 min	1.50E+05	2.10E+05	1.80E+05	0.344	5 min	3.30E+05	3.50E+05	3.40E+05	0.062
20 min	1.10E+05	1.50E+05	1.30E+05	0.485	10 min	2.70E+05	1.90E+05	2.30E+05	0.232
40 min	1.20E+05	1.10E+05	1.15E+05	0.538	20 min	9.00E+04	6.00E+04	7.50E+04	0.718

(Continued of Table A-22)

Reduction without freezing					Reduction after freezing				
Rep.6	Dup. 1	Dup. 2	Average	Reduction -log N/N_0	Rep.6	Dup. 1	Dup. 2	Average	Reduction -log N/N_0
					Before Freezing	3.50E+05	4.50E+05	4.00E+05	
Working solution:	4.30E+05	4.50E+05	4.40E+05		Frozen	3.90E+05		3.90E+05	0.011
Original	4.22E+05	4.41E+05	4.32E+05		Original	3.82E+05		3.82E+05	
5 min	3.20E+05	2.90E+05	3.05E+05	0.151	5 min	3.10E+05	3.50E+05	3.30E+05	0.064
10 min	2.30E+05	2.30E+05	2.30E+05	0.273	10 min	2.40E+05	2.20E+05	2.30E+05	0.220
20 min	1.90E+05	1.90E+05	1.90E+05	0.356	20 min	2.60E+05	1.80E+05	2.20E+05	0.240
40 min	2.00E+05	2.03E+05	2.02E+05	0.331	40 min	2.07E+05		2.07E+05	0.266
					Rep.7				
					Before. Freezing	2.90E+05	3.00E+05	2.95E+05	
					Frozen	2.60E+05	3.00E+05	2.80E+05	0.023
					Original	2.55E+05	2.94E+05	2.75E+05	
					5 min	2.20E+05	2.70E+05	2.45E+05	0.049
					10 min	1.80E+05	1.40E+05	1.60E+05	0.234
					20 min	1.00E+05	9.00E+04	9.50E+04	0.461
					40 min	9.00E+04	7.75E+04	8.38E+04	0.516

Note: Dup. = Duplicate; Rep.= Replicate

Table A-23. *Bacillus megaterium* reduction at 4.0 mg Cl_2/L.

Reduction without freezing					Reduction after freezing				
Rep.1	Dup. 1	Dup. 2	Average	Reduction -log N/N_0		Dup. 1	Dup. 2	Average	Reduction -log N/N_0
Working solution:	9.70E+05	1.05E+06	1.01E+06						
Original	6.47E+05	7.00E+05	6.73E+05		Frozen	9.70E+05	1.10E+06	1.04E+06	
5 min	3.30E+05	3.80E+05	3.55E+05	0.278	Original	5.72E+05	6.49E+05	6.11E+05	
10 min	2.70E+05	2.80E+05	2.75E+05	0.389	5 min	2.70E+05		2.70E+05	0.35
20 min	3.50E+04	4.00E+04	3.75E+04	1.254	10 min	2.00E+05	2.40E+05	2.20E+05	0.43
40 min	3.00E+03	2.50E+03	2.75E+03	2.389	20 min	2.90E+04	3.10E+04	3.00E+04	1.32
Rep.2					40 min	2.00E+02	4.00E+02	3.00E+02	3.21
Working solution:	7.80E+05	9.10E+05	8.45E+05						
Original	5.20E+05	6.07E+05	5.63E+05		Frozen	7.80E+05	9.10E+05	8.45E+05	
5 min	2.20E+05	2.00E+05	2.10E+05	0.429	Original	5.20E+05	6.07E+05	5.63E+05	
10 min	8.10E+04	5.60E+04	6.85E+04	0.915	5 min	2.00E+05	2.20E+05	2.10E+05	0.44
20 min	6.00E+03	8.00E+03	7.00E+03	1.906	10 min	5.60E+04	8.10E+04	6.85E+04	0.87
40 min	2.00E+02	3.00E+02	2.50E+02	3.353	20 min	6.00E+03	8.00E+03	7.00E+03	1.88
Rep.3					40 min	2.00E+02	3.00E+02	2.50E+02	3.31
Working solution:	7.20E+05	6.90E+05	7.05E+05		Before Freezing	9.60E+05	9.50E+05	9.55E+05	
Original	4.35E+05	4.17E+05	4.26E+05		Frozen	9.70E+05	1.05E+06	1.01E+06	-0.024
5 min	2.70E+05	2.20E+05	2.45E+05	0.240	Original	6.47E+05	7.00E+05	6.73E+05	
10 min	1.10E+05	1.00E+05	1.05E+05	0.608	5 min	3.80E+05	3.30E+05	3.55E+05	0.28
20 min	5.00E+03	6.00E+03	5.50E+03	1.889	10 min	2.80E+05	2.70E+05	2.75E+05	0.39

(*Contimued* of Table A-23)

Reduction without freezing					Reduction after freezing				
Rep.3	Dup. 1	Dup. 2	Average	Reduction -log N/N_0		Dup. 1	Dup. 2	Average	Reduction -log N/N_0
					20 min	3.50E+04	4.00E+04	3.75E+04	1.25
					40 min	3.00E+02	2.50E+02	2.75E+02	3.39
Rep.4									
Working solution:	4.20E+05	4.70E+05	4.45E+05						
Original	2.63E+05	2.95E+05	2.79E+05		Frozen	3.70E+05		3.70E+05	
5 min	1.30E+05	1.40E+05	1.35E+05	0.315	Original	2.34E+05		2.34E+05	
10 min	3.20E+04	3.80E+04	3.50E+04	0.902	5 min	1.80E+05	1.15E+05	1.48E+05	0.20
20 min	1.00E+03	9.00E+02	9.50E+02	2.468	10 min	5.30E+04	6.00E+04	5.65E+04	0.62
40 min	5.00E+01	7.00E+01	6.00E+01	3.668	20 min	4.60E+03	6.00E+03	5.30E+03	1.65
					40 min	8.00E+01	1.10E+02	9.50E+01	3.39

(Continued of Table A-23)

Bacillus reduction without freezing				
Rep.5	Dup. 1	Dup. 2	Ave.	Reduction -log N/N_0
Working solution:	3.90E+05	4.50E+05	4.20E+05	
Original	3.63E+05	4.19E+05	3.91E+05	
5 min	3.00E+05	2.95E+05	2.98E+05	0.119
10 min	1.10E+05	1.22E+05	1.16E+05	0.528
20 min	1.96E+04		1.96E+04	1.300
40 min	9.50E+02		9.50E+02	2.615
Rep.6				
Working solution:	4.20E+05	4.70E+05	4.45E+05	
Original	2.63E+05	2.95E+05	2.79E+05	
5 min	1.30E+05	1.40E+05	1.35E+05	0.315
10 min	3.20E+04	3.80E+04	3.50E+04	0.902
20 min	1.00E+03	9.00E+02	9.50E+02	2.468
40 min	5.00E+01	7.00E+01	6.00E+01	3.668
Rep.7				
Working solution:	6.50E+05	6.20E+05	6.35E+05	
Original	6.25E+05	5.96E+05	6.11E+05	
5 min	1.80E+05	1.90E+05	1.85E+05	0.519
10 min	1.20E+05	1.40E+05	1.30E+05	0.672
20 min	1.00E+04	1.70E+04	1.35E+04	1.655
40 min	4.70E+02	4.10E+02	4.40E+02	3.142

Note: Dup. = Duplicate; Rep.= Replicate

Table A-24. *Bacillus megaterium* reduction at 6.0 mg Cl_2/L.

Reduction without freezing					Reduction after freezing				
Rep.1	Dup. 1	Dup. 2	Ave.	Reduction -log N/N_0		Dup. 1	Dup. 2	Ave.	Reduction -log N/N_0
Working solution	2.60E+05	2.40E+05	2.50E+05		Before Freezing	4.50E+05	3.70E+05	4.10E+05	
Original	2.30E+05	2.12E+05	2.21E+05		Frozen	4.10E+05	3.90E+05	4.00E+05	0.011
5 min	1.30E+05	1.10E+05	1.20E+05	0.265	Original	3.86E+05	3.67E+05	3.76E+05	
10 min	1.20E+04		1.20E+04	1.265	5 min	1.70E+05	2.40E+05	2.05E+05	0.414
20 min	7.50E+01		7.50E+01	3.469	10 min	2.00E+04	2.50E+04	2.25E+04	1.224
					20 min	7.00E+01		7.00E+01	3.731
Rep.2									
Working solution	6.50E+05	6.20E+05	6.35E+05		Before Freezing	3.70E+05	5.50E+05	4.60E+05	
Original	6.12E+05	5.84E+05	5.98E+05		Frozen	4.40E+05	3.80E+05	4.10E+05	0.050
2.5 min	1.80E+05	2.70E+05	2.25E+05	0.425	Original	4.14E+05	3.58E+05	3.86E+05	
5 min	1.80E+05	1.07E+05	1.44E+05	0.620	5 min	1.55E+05	1.80E+05	1.68E+05	0.362
10 min	3.10E+04	2.60E+04	2.85E+04	1.322	10 min	6.00E+03	7.60E+03	6.80E+03	1.754
20 min	8.00E+01	7.50E+01	7.75E+01	3.888	20 min	3.00E+01		3.00E+01	4.109
Rep.3									
Working solution	3.60E+05	4.50E+05	4.05E+05		Before Freezing	3.60E+05	4.50E+05	4.05E+05	
Original	3.39E+05	4.24E+05	3.82E+05		Frozen	3.90E+05		3.90E+05	
2.5 min	1.50E+05	1.50E+05	1.50E+05	0.406	Original	3.66E+05		3.66E+05	
5 min	1.05E+05	8.00E+04	9.25E+04	0.615	2.5 min	1.70E+05	1.90E+05	1.80E+05	0.309

(*Continued* of Table A-24)

Reduction without freezing					Reduction after freezing				
Rep.3	Dup. 1	Dup. 2	Ave.	Reduction -log N/N$_0$		Dup. 1	Dup. 2	Ave.	Reduction -log N/N$_0$
10 min	1.00E+04	8.00E+03	9.00E+03	1.627	5 min	5.00E+04	3.00E+04	4.00E+04	0.962
20 min	1.05E+02	1.10E+02	1.08E+02	3.550	10 min	2.00E+03	1.50E+03	1.75E+03	2.321
					15 min		1.00E+02	1.00E+02	3.564
					20 min.	3.00E+00	3.33E+00	3.17E+00	5.064
Rep.4									
Working solution	4.30E+05	4.50E+05	4.40E+05		Pre. Freezing	2.90E+05	3.00E+05	2.95E+05	
Original	4.05E+05	4.24E+05	4.15E+05		Frozen	3.00E+05	2.60E+05	2.80E+05	
2.5 min	1.80E+05	2.70E+05	2.25E+05	0.265	Original	2.82E+05	2.45E+05	2.64E+05	
5 min	5.00E+04	6.00E+04	5.50E+04	0.877	2.5 min	1.30E+05	1.20E+05	1.25E+05	0.324
10 min	5.50E+03	6.50E+03	6.00E+03	1.839	5 min	4.50E+04	6.75E+04	5.63E+04	0.671
20 min	8.10E+02		8.10E+02	2.709	10 min	2.00E+03	1.70E+03	1.85E+03	2.154
					15 min.	4.00E+01	4.50E+01	4.25E+01	3.792
					20 min	7.25E+00	1.00E+00	4.13E+00	4.805

Note: Dup. = Duplicate; Rep.= Replicate

2.4. UV test for *Bacillus megaterium* reduction

Table A-25. *Bacillus megaterium* reduction without freezing (@-15°C)

Rep. 1	Fluence	E = 0.1006, Sample Absorbance: 0. 206 and Petri factor: 0.975			
	(mJ/cm^2)	R.1.1	R. 1.2	Ave.	Reduction
Original		5.20E+05	6.10E+05	5.65E+05	(-log(N/N$_0$)
3 min.	10.24	1.07E+05	1.30E+05	1.19E+05	0.678
7 min	23.89	2.20E+04	2.10E+04	2.15E+04	1.420
10 min	34.13				
13 min.	44.37		4.00E+01	4.00E+01	4.150
Rep. 2		E = 0.1007, Sample Absorbance: 0.396 and Petri factor: 0.975			
Time	Fluence	R.2.1	R 2.2		
Original		9.30E+05	1.05E+06	9.90E+05	
3 min.	10.25	1.80E+05	2.60E+05	2.20E+05	0.653
7 min	23.92	1.90E+04	4.60E+04	3.25E+04	1.484
10 min	34.17	3.00E+02	3.30E+02	3.15E+02	3.497
13 min.	44.42	6.00E+01	8.60E+01	7.30E+01	4.132
Rep. 3		E = 0.1166, Sample Absorbance: 0. 416 and Petri factor: 0.975			
Time	Fluence	R.3.1	R 3.2		
Original		9.00E+05	1.05E+06	9.75E+05	
3 min.	11.87	1.80E+05	1.00E+05	1.40E+05	0.843
7 min	27.69	5.00E+03	2.00E+03	3.50E+03	2.445
10 min	39.56	2.20E+02		2.20E+02	3.647
13 min.	51.43	7.80E+01	7.20E+01	7.50E+01	4.114
Rep.4		E = 0.1166, Sample Absorbance: 0. 184 and Petri factor: 0.915			
Time	Fluence	R.4.1	R 4.2		
Original		1.70E+05	1.70E+05	1.70E+05	
4'12"	20	1.42E+04	1.38E+04	1.40E+04	1.084
8'24"	40	4.00E+02	3.70E+02	3.85E+02	2.645
12'37"	60	4.10E+01	3.50E+01	3.80E+01	3.651
16'49"	80	2.80E+00	2.00E+00	2.40E+00	4.850

Note: In this experiment, the water factor was 0.6029 and the divergence factor was 0.962.

(Continued of Table A-25)

Rep. 5	Fluence	E = 0.1142, Sample Absorbance: 0. 206 and Petri factor: 0.93			
Time		R.5.1	R. 5.2	Ave.	Reduction
Original		2.70E+05	2.40E+05	2.55E+05	
4'14"	20	2.30E+04	2.40E+04	2.35E+04	1.035
8'29"	40	2.10E+02	2.00E+02	2.05E+02	3.095
12'43"	60	1.80E+01	1.00E+01	1.40E+01	4.260
16'57"	80	1.50E+01	1.68E+01	1.59E+01	4.205
Rep. 6	Fluence	E = 0.1283, Sample Absorbance: 0. 251 and Petri factor: 0.93			
Time		R.6.1	R 6.2		
Original		2.40E+05	2.50E+05	2.45E+05	
3'57"	20	2.58E+04	2.20E+04	2.39E+04	1.010
11'50"	60	1.00E+01	3.00E+01	2.00E+01	4.088
15'47"	80	2.00E+00	1.00E+01	6.00E+00	4.611
Rep. 7	Fluence	E = 0.1283, Sample Absorbance: 0. 251 and Petri factor: 0.93			
Time		R.7.1	R 7.2		
Original		2.40E+05	2.50E+05	2.45E+05	
3'57"	20	1.60E+04	1.90E+04	1.75E+04	1.146
7'53"	42.6	2.00E+02	3.00E+02	2.50E+02	2.991
11'50"	60	4.00E+01	4.00E+01	4.00E+01	3.787
15'47"	80	5.00E+00		5.00E+00	4.690

Table A-26. *Bacillus megaterium* reduction after freezing (@-15°C)

Rep. 1	Fluence	E = 0.1006, Sample Absorbance: 0. 405 and Petri factor: 0.975			
	(mJ/cm^2)	R.1.1	R 1.2	Ave.	Reduction
Original		6.50E+05	7.00E+05	6.75E+05	(-log(N/N$_0$))
3 min.	10.24	1.00E+05	1.40E+05	1.20E+05	0.75
7 min	23.89	5.10E+03	5.90E+03	5.50E+03	2.09
10 min	34.13	9.00E+02	1.05E+03	9.75E+02	2.84
13 min.	44.37	9.20E+02	9.80E+02	9.50E+02	2.85
Rep. 2		E = 0.1007, Sample Absorbance: 0.394 and Petri factor: 0.975			
Time	Fluence	R.2.1	R 2.2		
Original		7.20E+05	9.30E+05	8.25E+05	(-log(N/N$_0$))
3 min.	10.25	2.00E+05	1.40E+05	1.70E+05	0.69
7 min	23.92	6.60E+03	8.50E+03	7.55E+03	2.04
10 min	34.17	1.30E+03	1.60E+03	1.45E+03	2.76
13 min.	44.42	8.10E+02	7.90E+02	8.00E+02	3.01
Rep. 3		E = 0.1160, Sample Absorbance: 0. 436 and Petri factor: 0.975			
Time	Fluence	R.3.1	R 3.2		
Original		7.10E+05	7.70E+05	7.40E+05	(-log(N/N$_0$))
5'9"	20	4.20E+04	4.80E+04	4.50E+04	1.22
10'18"	40	1.18E+03	1.20E+03	1.19E+03	2.79
15'26"	60	3.10E+02	4.40E+02	3.75E+02	3.30
20'35"	80	1.20E+02		1.20E+02	3.79
Rep.4		E = 0.1166, Sample Absorbance: 0. 184 and Petri factor: 0.915			
Time	Fluence	R.4.1	R 4.2		
Original		8.10E+05	8.80E+05	8.45E+05	(-log(N/N$_0$))
5'17"	20	4.50E+04	3.90E+04	4.20E+04	1.30
10'34"	40	8.00E+02	1.20E+03	1.00E+03	2.93
15'51"	60	4.60E+02	5.40E+02	5.00E+02	3.23
21"7"	80	2.60E+02		2.60E+02	3.51

Note: In this experiment, the water factor was 0.6029 and the divergence factor was 0.962.

(Continued of Table A-26)

Rep. 5	Fluence	E = 0.1134, Sample Absorbance: 0. 228 and Petri factor: 0.93			
Time		R.5.1	R. 5.2	Ave.	Reduction
Original		2.80E+05	3.10E+05	2.95E+05	(-log(N/N_0)
5	20	1.00E+04	1.40E+04	1.20E+04	1.39
10'34"	40	4.60E+02	4.40E+02	4.50E+02	2.82
15'51"	60	3.00E+02	3.60E+02	3.30E+02	2.95
20'21"	80	2.00E+02	1.90E+02	1.95E+02	3.18
Rep. 6	Fluence	E = 0.1284, Sample Absorbance: 0. 271 and Petri factor: 0.93			
Time		R.6.1	R 6.2		
Original		3.60E+05	3.00E+05	3.30E+05	(-log(N/N_0)
4'4"	20	4.10E+04	4.50E+04	4.30E+04	0.89
8'9"	40	8.00E+02	6.80E+02	7.40E+02	2.65
12'13"	60	2.30E+02	2.00E+02	2.15E+02	3.19
16'18"	80	1.80E+02	1.20E+02	1.50E+02	3.34
Rep. 7	Fluence	E = 0.1284, Sample Absorbance: 0. 271 and Petri factor: 0.93			
Time		R.7.1	R 7.2		
Original		3.40E+05	3.20E+05	3.30E+05	(-log(N/N_0)
4'2"	20	4.50E+04	4.05E+04	4.28E+04	0.89
8'3"	40	1.00E+03	1.40E+03	1.20E+03	2.44
12'5"	60	4.58E+02	5.00E+02	4.79E+02	2.84
16'13"	80	1.90E+02	1.67E+02	1.78E+02	3.27

Appendix B. Data Analysis for *E.coli* Reduction

1. Fractional factorial design

1.1. m-FC method

Table B-1. Summary of regression analysis for m-FC method.

Regression Statistics	
Multiple R	0.895
R Square	0.801
Adjusted R Square	0.702
Standard Error	0.338
Observations	16

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	5	4.593	0.919	8.058	0.003
Residual	10	1.140	0.114		
Total	15	5.734			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.932	0.084	11.045	0.000	0.744	1.120
A	0.027	0.084	0.322	0.754	-0.161	0.215
B	0.455	0.084	5.388	0.000	0.267	0.643
C	0.208	0.084	2.469	0.033	0.020	0.396
D	-0.189	0.084	-2.245	0.049	-0.378	-0.001
E = AB	0.012	0.084	0.139	0.892	-0.176	0.200
AC	0.035	0.116	0.297	0.777	-0.250	0.319
AD	-0.003	0.116	-0.024	0.982	-0.288	0.282
BC	0.243	0.116	2.084	0.082	-0.042	0.527
BD	-0.028	0.116	-0.243	0.816	-0.313	0.256
CD	0.032	0.116	0.272	0.795	-0.253	0.316
CE	0.087	0.116	0.750	0.481	-0.197	0.372
DE	0.005	0.116	0.042	0.968	-0.280	0.290

1.1.1 Estimate model for m-FC method

For m-FC method, the estimate model is:

$$Y = 0.932 + 0.455 \cdot X_B + 0.208 \cdot X_C - 0.189 \cdot X_D$$

Checking the residuals versus n-score and the main factors.

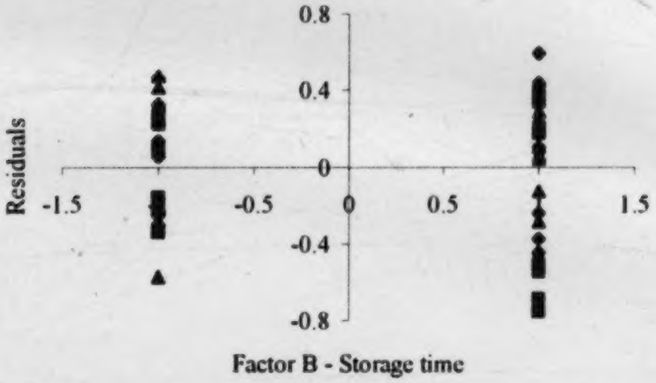


Figure A-1. Residuals vs. Storage time

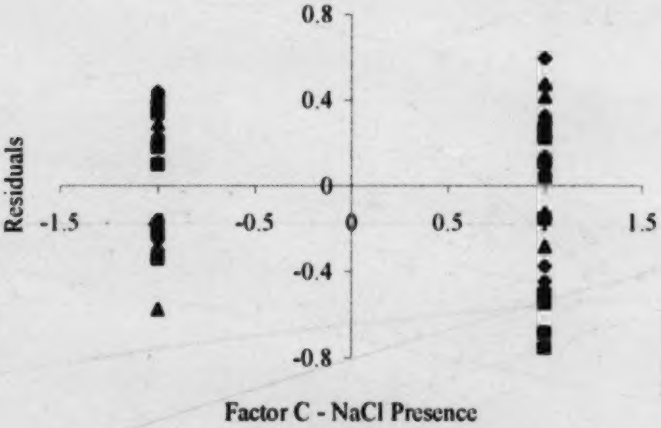


Figure A-2. Residuals vs. Nacl Presence

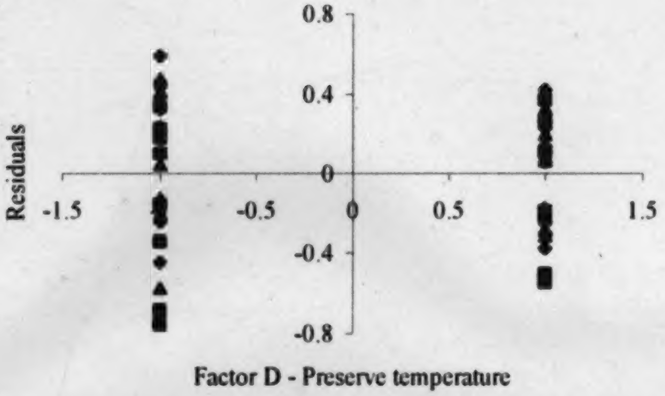


Figure A-3. Residuals vs. Preserve temperature

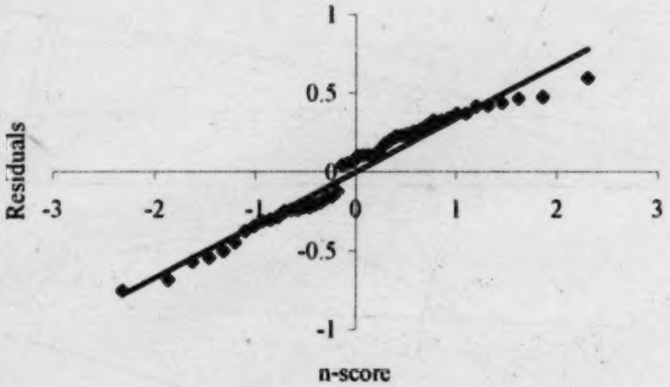


Figure A-4. Residuals vs. n-score

From checking the residuals, the residuals had the constant variances versus the three factors and also the residuals fit the normal distribution.

1.2. Modified m-FC method

Table B-2. Summary of regression analysis for modified m-FC method.

Regression Statistics	
Multiple R	0.894
R Square	0.799
Adjusted R Square	0.698
Standard Error	0.306
Observations	16

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	5	3.728	0.746	7.949	0.003
Residual	10	0.938	0.094		
Total	15	4.665			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.850	0.077	11.105	0.000	0.680	1.021
A	0.003	0.077	0.042	0.967	-0.167	0.174
B	0.407	0.077	5.321	0.000	0.237	0.578
C	0.208	0.077	2.721	0.022	0.038	0.379
D	-0.153	0.077	-1.998	0.074	-0.324	0.018
E = AB	0.016	0.077	0.206	0.841	-0.155	0.186
AC	0.045	0.108	0.418	0.690	-0.218	0.308
AD	-0.010	0.108	-0.091	0.931	-0.273	0.253
BC	0.210	0.108	1.954	0.099	-0.053	0.473
BD	0.020	0.108	0.189	0.857	-0.243	0.284
CD	0.005	0.108	0.049	0.963	-0.258	0.268
CE	0.097	0.108	0.899	0.403	-0.166	0.360
DE	-0.009	0.108	-0.086	0.934	-0.273	0.254

1.2.1 Estimate model for modified m-FC method

For Modified m-FC method, the estimate model is:

$$Y = 0.85 + 0.407 \cdot X_B + 0.208 \cdot X_C - 0.153 \cdot X_D$$

Checking the residuals versus n-score and the main factors.

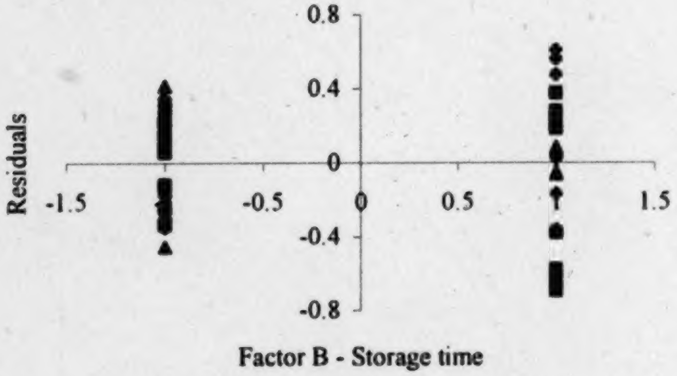


Figure A-5. Residuals vs. storage time

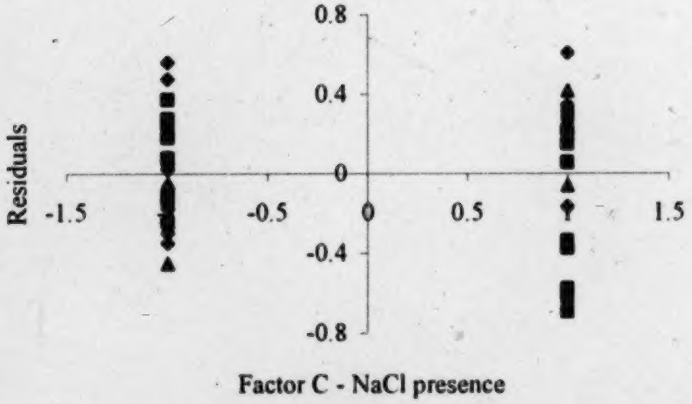


Figure A-6. Residuals vs. NaCl presence

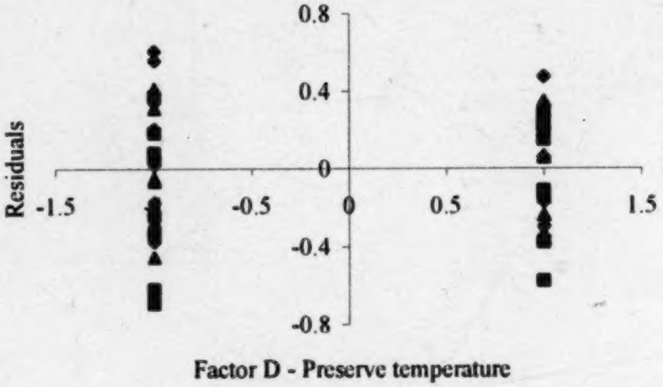


Figure A-7. Residuals vs. Preserve temperature

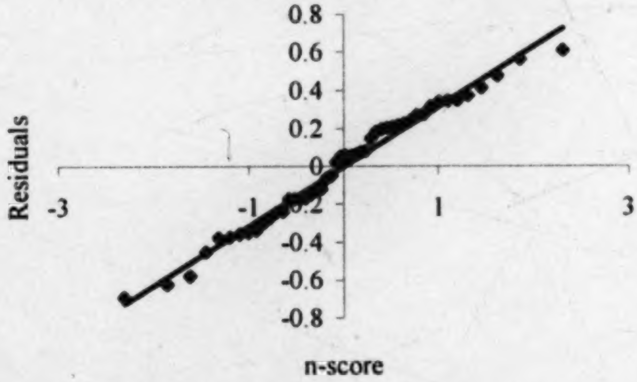


Figure A-8. Rsiduals vs. n-score

From checking the residuals, the residuals had the constant variances versus the three factors and also the residuals fit the normal distribution.

2. *E.coli* reduction in different growth phase

2.1. Comparing the different reduction between two methods (after freezing)

Table B-3. t-test for the different reduction of two method (after freezing).

Rep.1		Rep.2		Rep.3		Rep.4	
Time (hrs)	Difference in reduction (-log N/N_0)	Time (hrs)	Difference in reduction (-log N/N_0)	Time (hrs)	Difference in reduction (-log N/N_0)	Time (hrs)	Difference in reduction (-log N/N_0)
0.5	0.10	0.5	0.02	2	0.15	24	0.60
2	-0.27	1	0.04	4	-0.10	48	0.37
4	0.15	3	0.00	7	-1.59	72	0.42
8	0.26	7	0.00	24	0.05	96	0.28
24	0.02	24	-0.11	30	0.41	120	0.10
72	0.41	30	-0.08	48	0.22	168	0.01
120	-0.08	48	0.00	72	0.38	192	0.18
192	-0.06	72	-0.02	96	0.59	216	0.01
240	-0.23	96	0.00	120	0.44	240	0.10
384	0.53	120	-0.11	144	0.32	312	0.25
		144	-0.11	168	0.58		
		168	0.19	196	0.89		
		192	0.53	216	0.34		
		264	1.01	264	-0.04		
		384	0.36	288	-0.02		

Data analysis:

Average of the difference (-log N/N_0): 0.18

Sample Variance: 0.276

$t = 4.48 > t_{(0.05, 49)} = 2.02$

So, two methods have difference *E.coli* reduction.

2.2. Comparing two methods in test *E.coli* density in stationary growth phase.

Using "General linear model" in SPSS to analyze the *E.coli* number in stationary growth phase and comparing the two test methods. The output is showed in following.

Table B-4. Univariate Analysis of Variance

(Between-Subjects Factors_

		N
Growth time (hours)	24.00	8
	30.00	4
	48.00	6
	72.00	8
	96.00	6
	120.00	8
	144.00	4
	168.00	6
	192.00	6
	196.00	2
	216.00	4
	240.00	4
	264.00	4
	288.00	2
	312.00	2
	384.00	4
Test methods	m-FC	39
	Mm-FC	39

Table B-5 Tests of Between-Subjects Effects (Dependent Variable: reduction ($-\log N/N_0$))

Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	78.625(a)	31	2.536	6.999	0.000
Intercept	295.515	1	295.515	815.536	0.000
Time	78.625	15	5.242	14.466	0.000
Methods	0.000	1	0.000	0.000	0.996
Time * Methods	0.000	15	0.000	0.000	1.000
Error	16.668	46	0.362		
Total	374.215	78			
Corrected Total	95.294	77			

(a) R Squared = 0.825 (Adjusted R Squared = 0.707)

From the results, the two test methods have no significant difference in counting *E.coli* density without freezing.

3. *E.coli* reduction versus storage time

Using General Liner Model in "SPSS" program doing the Data analysis and determining the effects of three factors, storage time, test method, freezing temperature, to *E.coli* removal. The output of results is showed as in following.

Table B-6. Univariate Analysis of Variance
(Between-Subjects Factors)

		N
Freezing temperature	-35	65
	-15	67
	-5	46
Storage time (Days)	0	32
	2	29
	5	28
	10	32
	20	30
	30	27
Test methods	Mm-FC	89
	m-FC	89

Table B-7. Tests of Between-Subjects Effects (Dependent Variable: Removal ($-\log N/N_0$))

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	53.494(a)	35	1.528	15.720	0.000
Intercept	316.639	1	316.639	3256.744	0.000
Temperature	25.048	2	12.524	128.815	0.000
Time	17.737	5	3.547	36.486	0.000
Methods	4.428	1	4.428	45.548	0.000
Temperature * Time	4.591	10	0.459	4.722	0.000
Temperature * Methods	0.296	2	0.148	1.525	0.221
Time * Methods	0.265	5	0.053	0.546	0.741
Temperature * Time * Methods	0.324	10	0.032	0.333	0.971
Error	13.806	142	0.097		
Total	362.746	178			
Corrected Total	67.300	177			

(a) R Squared = 0.795 (Adjusted R Squared = 0.744)

From the results, three factors all had significant effect to *E.coli* reduction.

4. *E.coli* reduction versus freeze/thaw cycles

Using General Liner Model in "SPSS" program doing the Data analysis and determining the effects of three factors, Freezing cycles, test method and freezing temperature, to *E.coli* removal. The output of results is showed as in following.

Table B-8. Univariate Analysis of Variance (Between-Subjects Factors)

		N
Test methods	m-FC	52
	Mm-FC	55
Freezing temperature	-35.00	28
	-15.00	35
	-5.00	44
Freezing Cycle	1	22
	2	20
	3	18
	4	18
	5	17
	6	12

Table B-9. Tests of Between-Subjects Effects (Dependent Variable: reduction)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	117.884(a)	34	3.467	22.613	0.000
Intercept	428.649	1	428.649	2795.686	0.000
Methods	1.201	1	1.201	7.832	0.007
Temperature	19.513	2	9.757	63.634	0.000
Cycle	72.936	5	14.587	95.139	0.000
Methods * Temperature	0.763	2	0.381	2.488	0.090
Methods * Cycle	0.127	5	0.025	0.165	0.974
Temperature * Cycle	3.624	10	0.362	2.364	0.018
Methods * Temperature * Cycle	0.360	9	0.040	0.261	0.983
Error	11.039	72	0.153		
Total	564.896	107			
Corrected Total	128.923	106			

(a) R Squared = 0.914 (Adjusted R Squared = 0.874)

5. *E.coli* reduction in spraying freezing test

Table B-10. Pair t-test in different spraying time for *E.coli* reduction in melted ice water (m-FC method)

	SPRAYING TIME (MINUTES)	
	15	45
Mean	0.368	0.343
Variance	0.011	0.017
Observations	4	4
Pearson Correlation	-0.323	
Hypothesized Mean Difference	0	
df	3	
t Stat	-0.263	
P(T<=t) one-tail	0.405	
t Critical one-tail	2.353	
P(T<=t) two-tail	0.810	
t Critical two-tail	3.182	

Because $|t| < t_{crit.}$, there no significant different in two spraying time.

Table B-11. Pair t-test in different spraying time for *E.coli* reduction in melted ice water (Modified m-FC method)

	Spraying time (minutes)	
	15	45
Mean	0.323	0.368
Variance	0.006	0.016
Observations	4	4
Pearson Correlation	0.308	
Hypothesized Mean Difference	0	
df	3	
t Stat	-0.705	
P(T<=t) one-tail	0.266	
t Critical one-tail	2.353	
P(T<=t) two-tail	0.532	
t Critical two-tail	3.182	

Because $|t| < t_{crit.}$, there no significant different in two spraying time.

Appendix C. Data Analysis for *Bacillus megaterium* Reduction

1. UV test

t-test for means comparison (a = 0.05)

Table C-1. Fluence at 20 mJ/cm^2

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Equal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	1.069	1.137	Mean	1.069	1.137
Variance	0.004	0.056	Variance	0.004	0.056
Observations	4	5	Observations	4	5
df	3	4	Pooled Variance	0.034	
F	0.0639		Hypothesized Mean Difference	0	
$P_{(F<=f)}$ one-tail	0.0238		df	7	
F Critical one-tail	0.1097		t Stat	-0.549	
F < F$_{Crit.}$, so two samples had equal variance			P(T<=t) one-tail	0.300	
			t Critical one-tail	1.895	
			P(T<=t) two-tail	0.600	
			t Critical two-tail	2.365	

t < t$_{Crit.}$, so two sample means are equal.

Table C-2. Fluence at 40 mJ/cm^2

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Equal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	2.910	2.725	Mean	2.910	2.725
Variance	0.055	0.035	Variance	0.055	0.035
Observations	3	5	Observations	3	5
df	2	4	Pooled Variance	0.042	
F	1.572		Hypothesized Mean Difference	0	
P(F<=f) one-tail	0.314		df	6	
F Critical one-tail	6.944		t Stat	1.237	
$F < F_{Crit.}$, so two samples had equal variance			P(T<=t) one-tail	0.131	
			t Critical one-tail	1.943	
			P(T<=t) two-tail	0.262	
			t Critical two-tail	2.447	

$t < t_{Crit.}$, so two sample means are equal.

Table C-3. Fluence at 60 mJ/cm^2

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Equal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	3.947	3.100	Mean	3.947	3.100
Variance	0.077	0.038	Variance	0.077	0.038
Observations	4	5	Observations	4	5
df	3	4	Pooled Variance	0.055	
F	2.023		Hypothesized Mean Difference	0	
P(F<=f) one-tail	0.253		df	7	
F Critical one-tail	6.591		t Stat	5.389	
$F < F_{Crit.}$, so two samples had equal variance			P(T<=t) one-tail	0.001	
			t Critical one-tail	1.895	
			P(T<=t) two-tail	0.001	
			t Critical two-tail	2.365	

$t > t_{Crit.}$, so two sample means are different.

Table C-4. Fluence at 80 mJ/cm^2

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Equal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	4.589	3.418	Mean	4.589	3.418
Variance	0.075	0.058	Variance	0.075	0.058
Observations	4	5	Observations	4	5
df	3	4	Pooled Variance	0.066	
F	1.298		Hypothesized Mean Difference	0	
$P_{(F<=f)}$ one-tail	0.390		df	7	
F Critical one-tail	6.591		t Stat	6.818	
$F < F_{Crit.}$, so two samples had equal variance			P(T<=t) one-tail	1.2E-04	
			t Critical one-tail	1.895	
			P(T<=t) two-tail	2.5E-04	
			t Critical two-tail	2.365	

$t > t_{Crit.}$, so two sample means are different.

2. Chlorination

Table C-5. Chlorination for *Bacillus megaterium* reduction

	Reduction (-log N/N_0)							
Chlorine conc.	5 minutes		10 minutes		20minutes		40 minutes	
	Without Freezing	With Freezing	Without Freezing	With Freezing	Without Freezing	With Freezing	Without Freezing	With Freezing
2 mg/L	0.263	0.073	0.410	0.244	0.395	0.356	0.648	0.293
	0.154	0.062	0.343	0.106	0.356	0.199	0.564	0.248
	0.201	0.064	0.213	0.142	0.318	0.332	0.216	0.499
	0.151	0.049	0.247	0.378	0.236	0.429	0.538	0.411
			0.344	0.232	0.485	0.718	0.331	0.266
			0.273	0.220	0.356	0.240		0.516
				0.234		0.461		
4 mg/L	0.278	0.354	0.389	0.432	1.254	1.321	2.389	3.306
	0.429	0.441	0.915	0.874	1.906	1.880	3.353	3.389
	0.240	0.278	0.608	0.389	1.889	1.254	3.668	3.392
	0.315	0.201	0.902	0.617	2.468	1.645	2.615	
	0.119		0.528		1.300		3.668	
	0.315		0.902		2.468		3.142	
	0.5186		0.6718		1.6554			
	2.5 minutes		5 minutes		10 minutes		20minutes	
6 mg/L	0.425	0.309	0.265	0.264	1.265	1.224	3.469	3.731
	0.406	0.324	0.620	0.362	1.322	1.754	3.888	4.109
	0.265		0.615	0.962	1.627	2.321	3.550	5.064
			0.877	0.671	1.839	2.154	2.709	4.805

t-test for means comparison (a = 0.10)

2.1. Free chlorine: 2.0 mg Cl_2/L

5 minutes:

t-Test: Paired Two Sample for Means		
	Without Freezing	With Freezing
Mean	0.192	0.06194
Variance	0.003	9.2E-05
Observations	4	4
Pearson Correlation	0.8589	
Hypothesized Mean Difference	0	
df	3	
t Stat	5.871	
P(T<=t) one-tail	0.005	
t Critical one-tail	1.638	
P(T<=t) two-tail	0.010	
t Critical two-tail	2.353	

t > $t_{Crit.}$, so two sample means are different.

10 minutes

F-Test Two-Sample for Variances			t-Test-test		
			Two-Sample Assuming Unequal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	0.305	0.222	Mean	0.305	0.222
Variance	0.005	0.007	Variance	0.005	0.007
Observations	6	7	Observations	6	7
df	5	6	Hypothesized Mean Difference	0	
F	0.715		df	11	
P(F<=f) one-tail	0.365		t Stat	1.863	
F Critical one-tail	0.294		P(T<=t) one-tail	0.045	
F > $F_{Crit.}$, so two samples had unequal variances			t Critical one-tail	1.363	
			P(T<=t) two-tail	0.089	
			t Critical two-tail	1.796	

t > $t_{Crit.}$, so two sample means are different.

20 minutes

F-Test Two-Sample for Variances			t-Test:		
			Two-Sample Assuming Equal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	0.358	0.391	Mean	0.358	0.391
Variance	0.007	0.030	Variance	0.007	0.030
Observations	6	7	Observations	6	7
df	5	6	Pooled Variance	0.019	
F	0.229		Hypothesized Mean Difference	0	
P(F<=f) one-tail	0.063		df	11	
F Critical one-tail	0.294		t Stat	-0.427	
$F < F_{Crit.}$, so two samples had equal variances			P(T<=t) one-tail	0.339	
			t Critical one-tail	1.363	
			P(T<=t) two-tail	0.677	
			t Critical two-tail	1.796	

$t < t_{Crit.}$, so two sample means are equal.

40 minutes

F-Test Two-Sample for Variances			t-Test:		
			Two-Sample Assuming Equal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	0.459	0.372	Mean	0.459	0.372
Variance	0.032	0.014	Variance	0.032	0.014
Observations	5	6	Observations	5	6
df	4	5	Pooled Variance	0.022	
F	2.264		Hypothesized Mean Difference	0	
P(F<=f) one-tail	0.197		df	9	
F Critical one-tail	3.520		t Stat	0.967	
$F < F_{Crit.}$, so two samples had equal variances			P(T<=t) one-tail	0.179	
			t Critical one-tail	1.383	
			P(T<=t) two-tail	0.359	
			t Critical two-tail	1.833	

$t < t_{Crit.}$, so two sample means are equal.

2.2. Free chlorine: 4.0 mg Cl_2/L

5 minutes

F-Test Two-Sample for Variances			t-Test:		
			Two-Sample Assuming Equal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	0.316	0.318	Mean	0.316	0.318
Variance	0.017	0.011	Variance	0.017	0.011
Observations	7	4	Observations	7	4
df	6	3	Pooled Variance	0.015	
F	1.575		Hypothesized Mean Difference	0	
P(F<=f) one-tail	0.381		df	9	
F Critical one-tail	5.285		t Stat	-0.027	
$F < F_{Crit.}$, so two samples had equal variances			P(T<=t) one-tail	0.489	
			t Critical one-tail	1.383	
			P(T<=t) two-tail	0.979	
			t Critical two-tail	1.833	

$t < t_{Crit.}$, so two sample means are equal.

10 minutes

F-Test Two-Sample for Variances			t-Test:		
			Two-Sample Assuming Unequal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	0.702	0.578	Mean	0.702	0.578
Variance	0.044	0.049	Variance	0.044	0.049
Observations	7	4	Observations	7	4
df	6	3	Hypothesized Mean Difference	0	
F	0.898		df	6	
P(F<=f) one-tail	0.415		t Stat	0.911	
F Critical one-tail	0.304		P(T<=t) one-tail	0.199	
$F > F_{Crit.}$, so two samples had unequal variances			t Critical one-tail	1.440	
			P(T<=t) two-tail	0.397	
			t Critical two-tail	1.943	

$t < t_{Crit.}$, so two sample means are equal.

20 minutes

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Equal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	1.849	1.525	Mean	1.849	1.525
Variance	0.244	0.085	Variance	0.244	0.085
Observations	7	4	Observations	7	4
df	6	3	Pooled Variance	0.191	
F	2.866		Hypothesized Mean Difference	0	
P(F<=f) one-tail	0.208		df	9	
F Critical one-tail	5.285		t Stat	1.181	
$F < F_{Crit.}$ so two samples had equal variances			P(T<=t) one-tail	0.134	
			t Critical one-tail	1.383	
			P(T<=t) two-tail	0.268	
			t Critical two-tail	1.833	

$t < t_{Crit.}$, so two sample means are equal.

40 minutes

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Unequal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	3.139	3.362	Mean	3.139	3.362
Variance	0.288	0.002	Variance	0.288	0.002
Observations	6	3	Observations	6	3
df	5	2	Hypothesized Mean Difference	0	
F	120.994		df	5	
P(F<=f) one-tail	0.008		t Stat	-1.010	
F Critical one-tail	9.293		P(T<=t) one-tail	0.179	
$F > F_{Crit.}$, so two samples had unequal variances			t Critical one-tail	1.476	
			P(T<=t) two-tail	0.359	
			t Critical two-tail	2.015	

$t < t_{Crit.}$, so two sample means are equal.

2.3. Free chlorine: 6.0 mg Cl_2/L

2.5 minutes

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Unequal Variances		
	Without Freezing	With Freezing		Without Freezing	With Freezing
Mean	0.365	0.316	Mean	0.365	0.316
Variance	0.008	0.000	Variance	0.008	0.000
Observations	3	2	Observations	3	2
df	2	1	Hypothesized Mean Difference	0	
F	64.718		df	2	
P(F<=f) one-tail	0.088		t Stat	0.964	
F Critical one-tail	49.500		P(T<=t) one-tail	0.218	
F > $F_{Crit.}$, so two samples had unequal variances			t Critical one-tail	1.886	
			P(T<=t) two-tail	0.437	
			t Critical two-tail	2.920	

t < $t_{Crit.}$, so two sample means are equal.

5 minutes

t-Test: Paired Two Sample for Means		
	Without Freezing	With Freezing
Mean	0.594	0.602
Variance	0.063	0.076
Observations	4	4
Pearson Correlation	0.399	
Hypothesized Mean Difference	0	
df	3	
t Stat	-0.054	
P(T<=t) one-tail	0.480	
t Critical one-tail	1.638	
P(T<=t) two-tail	0.960	
t Critical two-tail	2.353	

t < $t_{Crit.}$, so two sample means are equal.

10 minutes

t-Test: Paired Two Sample for Means		
	Without Freezing	With Freezing
Mean	1.513	1.863
Variance	0.073	0.238
Observations	4	4
Pearson Correlation	0.828	
Hypothesized Mean Difference	0	
df	3	
t Stat	-2.292	
P(T<=t) one-tail	0.053	
t Critical one-tail	1.638	
P(T<=t) two-tail	0.106	
t Critical two-tail	2.353	

$|t| > t_{Crit.}$ one-tail, so with freezing had higher reduction rate than without freezing.

20 minutes

t-Test: Paired Two Sample for Means		
	Without Freezing	With Freezing
Mean	3.404	4.427
Variance	0.247	0.378
Observations	4	4
Pearson Correlation	-0.402	
Hypothesized Mean Difference	0	
df	3	
t Stat	-2.192	
P(T<=t) one-tail	0.058	
t Critical one-tail	1.638	
P(T<=t) two-tail	0.116	
t Critical two-tail	2.353	

$|t| > t_{Crit.}$ one-tail, so with freezing had higher reduction rate than without freezing.